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Andy H. Choi
Besim Ben-Nissan *Editors*

Marine-Derived Biomaterials for Tissue Engineering Applications

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The Springer Series in Biomaterials Science and Engineering addresses the manufacture, structure and properties, and applications of materials that are in contact with biological systems, temporarily or permanently. It deals with many aspects of modern biomaterials, from basic science to clinical applications, as well as host responses. It covers the whole spectrum of biomaterials – polymers, metals, glasses and ceramics, and composites/hybrids – and includes both biological materials (collagen, polysaccharides, biological apatites, etc.) and synthetic materials. The materials can be in different forms: single crystals, polycrystalline materials, particles, fibers/wires, coatings, non-porous materials, porous scaffolds, etc. New and developing areas of biomaterials, such as nano-biomaterials and diagnostic and therapeutic nanodevices, are also focuses in this series. Advanced analytical techniques that are applicable in R & D and theoretical methods and analyses for biomaterials are also important topics. Frontiers in nanomedicine, regenerative medicine and other rapidly advancing areas calling for great explorations are highly relevant.

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Marine-Derived Biomaterials for Tissue Engineering Applications

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Preface

Highly functional architectural structures with interconnecting open pores can be easily uncovered from within the marine environment. Marine organisms are organized and produced from materials that possess a wide range of properties and characteristics, and in particular, their high mechanical strength and chemical composition warrant their potential use in the biomedical arena such as bone graft applications, regardless if they are used in their original form or converted to materials more suitable for implantation in humans.

More importantly, the task of utilizing our natural marine resources in a sustainable manner creates an extremely motivating platform for the research and development of new biomaterials that brings together both economic and environmental benefits. Consequently, an ever-increasing number of compounds of various categories are being isolated from marine or aquatic organisms and converted to products intended for biomedical applications such as controlled drug delivery devices and tissue engineering scaffolds.

During the last past 20 years or so, topics such as soft and hard tissue engineering, the discovery of a new generation of organic molecules, and more efficient pharmaceutical drug delivery systems with improved properties have been the major emphases in the field of marine-based structures. An ever-increasing number of studies have been conducted focusing on biopolymers and proteins generated by marine organisms for applications in the medical arena.

Opportunity has been created by the applications of ready-made inorganic and organic marine skeletons, as they could potentially present one of the simplest answers to important questions hindering the future research and development of regenerative medicine in orthopedics and dentistry such as providing available and abundant sources of osteopromotive analogues and biomineralization proteins and a richness of framework designs and devices.

Currently, a rising number of materials and compounds such as proteins and calcium carbonate are being isolated from marine structures and used for medical purposes. Furthermore, converted coral skeletons and coralline apatites are perfect examples in tissue engineering, as these converted materials have displayed considerable clinical success when used as templates or scaffolds for the reconstruction

of soft and hard tissues. As a result of this success, researchers are encouraged to investigate other marine organisms with improved biological and/or mechanical properties. These unique three-dimensional marine structures are capable of supporting the growth and differentiation of stem cell progenitors into bone cells.

Corals and marine sponge skeletons have been established to support self-sustaining musculoskeletal tissues and the extracts of nacre seashell organic matrixes and sponging collagen can encourage bone mineralization. This notion was reinforced by the fact that endodermal cells generate bone morphogenetic protein molecules into the developing skeleton. Moreover, the regenerative signaling proteins in bone therapeutics are also discovered during the early developmental stages of marine sponges and they are influential in the instigation of stem cells in cnidarians.

Likewise, the composition of the scaffold material has also been recognized to be of vital importance to the establishment of stem cell activities due to the fact that they rely on the extracellular fabric to guide their subsequent evolution and development as well as for life support. This can be achieved through the utilization of naturally occurring biomaterials including their reconstituted forms and derivatives. The primary intentions of regenerative medicine are to create micro-environments which are more ideal at regulating tissue development and formation, enhancement in stem cell processing, and to create less invasive transplantation modulus with site-specific targeting properties.

This book comprises 21 chapters written by experts in the fields of tissue engineering and drug delivery from all over the world. Each chapter provides an in-depth analysis into the use of marine organisms as templates for tissue reconstruction. Moreover, up-to-date findings into the use of marine materials such as coral exoskeletons and marine shells for drug delivery systems will also be covered.

This book is divided into four major parts. The first part covers biomimicry, evolution, and applications of marine structures such as coral, sponge, sea urchin, sponge nacre, and foraminifera for bioactive bone scaffolding materials and tissue engineering. The second part discusses the production of calcium phosphates bioceramics from marine sources such as fish bones, fish scales, corals, cuttlefish bones, shellfish, and marine algae. It also covers the utilization of chitosan, hydroxyapatite, and diatoms for bone tissue engineering. The third part focuses on the use of marine sources and other marine products for potential pharmaceutical applications such as anti-HIV, anti-inflammatory, anti-cancer, anti-obesity, and anti-diabetes. It also reviews the safety, quality, efficacy, and recent advances on its applications in dentistry and orthopedics. The last part discusses the design criteria of an ideal scaffold for both soft and hard tissue engineering. It also examines the clinical application of different marine-derived materials such as porifera, marine sponges, sea urchin, marine fauna, marine algae, marine polysaccharides, and red sea algae as well as their biological activity in enhancing bone repair and regeneration *in vitro* and *in vivo*.

Finally, I would like to thank all the authors for their time and great contribution to this informative book. I would also like to thank my great family for their support throughout this endeavor. Also, a very special thank you to my mentor and co-editor, Prof. Besim Ben-Nissan, for his teachings and friendship for nearly two decades. Finally, I would like to acknowledge the people at Springer Publishing for making this book possible.

Sydney, Australia Andy H. Choi

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About the Editors

Dr. Andy H. Choi is an early career researcher who received his Ph.D. from the University of Technology Sydney (UTS) in Australia in 2004 on the use of computer modeling and simulation known as finite element analysis (FEA) to examine the biomechanical behavior of implants installed into a human mandible. After completing his Ph.D., he expanded his research focus from FEA to sol–gel synthesis of multifunctional calcium phosphate nanocoatings and nanocomposite coatings for dental and biomedical applications.

In late 2010, Dr. Choi was successfully awarded the internationally competitive Endeavour Australia Cheung Kong Research Fellowship Award and undertook postdoctoral training at the Faculty of Dentistry of the University of Hong Kong focusing on the application of FEA in dentistry and the development of calcium phosphate nanobioceramics.

He has served as an Associate Editor for the *Journal of the Australian Ceramic Society* and on the editorial boards for a number of dentistry, nanotechnology, and orthopedics journals. To date, Dr. Choi has authored over 50 publications including 3 books and 26 book chapters on calcium phosphate, nanobiomaterial coatings, sol–gel technology, marine structures, drug delivery, tissue engineering, and finite element analysis in nanomedicine and dentistry.

Prof. Besim Ben-Nissan has B.Eng. in Metallurgical Engineering (ITU), M.Sc. degree in Ceramic Engineering, and Ph.D. in Mechanical and Biomedical Engineering both from the UNSW Australia.

During his formative years, Prof. Ben-Nissan worked on Titanium and its alloys and Magnesium alloy development and casting technologies and their properties for both engineering and medical applications. Over the last four decades, Professor Ben-Nissan has worked and contributed to the biomedical materials, implant design, production and analysis of various advanced ceramics, nanocoated sol–gel developed thin films, coated orthopedic and dental implants, anti-microbial slow drug delivery devices and methods, marine structures for clinical applications, biomechanics and finite element analysis of medical materials and engineering structures.

He has successfully developed materials for implant technology such as ceramic knee prosthesis, calcium-phosphate-based bioactive materials, bone graft production and bio-composites, and conducted research on biomechanics and modeling such as jaw bone, knee and hip joints, reliability and implant design modular zirconia ceramic knee prosthesis, femoral head and taper stresses and artificial ocular implants and bionic eye and recently on 3D printing of bioceramics and metallic implants and anti-microbial multifunctional coatings for drug delivery which are supported by the European Commission and the Australian Academy of Science research grants.

Since year 2000 he has published over 200 fully refereed papers in journals, and a book and 43 book chapters. He edited a book on Calcium phosphates and working on a second one on the use of Marine Structures in the Biomedical field. He is the Editor of the *Journal of the Australasian Ceramic Society*. He was awarded by the Australian Ceramic Society's prestigious award for his contributions to the "Ceramics Research & Development and Education in Australia". For his research on multifunctional nanocoatings he also received "The Future Materials Award".

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Chapter 1
Thoughts and Tribulations on Bioceramics and Marine Structures

Besim Ben-Nissan, Andy H. Choi, David W. Green, Ipek Karacan, Sibel Akyol and Sophie Cazalbou

Abstract Marine organisms are structured and constituted by materials with a vast range of properties and characteristics that may justify their potential application within the biomedical field. This is demonstrated by the biological effectiveness of marine structures such as corals and shells and sponge skeletons to house self-sustaining musculoskeletal tissues and their ability to promote bone formation though the use of extracts from sponging and nacre seashells. The design and composition of marine structures have been instrumental in the solving vital problems in regenerative medicine through the introduction of basic remedies that provides frameworks and highly accessible sources of osteopromotive analogues of bioceramic monoliths, nanofibres, micro and macrospheres. The clinical success of any future regenerative implants will be dependent on the production of highly proficient scaffolds that biologically operates at the nano-, micro- and macroscopic levels. Moreover, the implant will also need to coordinate, assemble, and organize cells into tissues as well as releasing encapsulated chemical signals in a targeted way and convey them into the body. As a result, an increasing number of different types of compounds are being isolated from aquatic organisms and transformed into products for health applications, including controlled drug delivery and tissue engineering devices. Despite the fact that they are extremely effective, the development of these materials has their drawbacks that needs be addressed. This chapter reviews the current bioceramics and natural marine structures including their structure, morphology, and applications in regenerative medicine, bone grafts, and drug delivery. In addition, the extraction of

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biological materials such as proteins from marine materials will also be discussed. An example of this specific biomimicry is provided by filtering the microskeleton of Foraminifera and coralline microspheres. New selected strategies based on our research as well as the works of others concerning the engineering of new bone tissues based on biomimicry will be also examined.

Keywords Hydroxyapatite · Coral · Sponge · Sea urchin · Nacre · Hydrothermal conversion · Bioceramics

1.1 Introduction

There is a constant and bountiful diversification of lipids, carbohydrates, pharmaceutical and therapeutic proteins. This has been stimulated by an increase in knowledge of molecular events associated with tissues and organs in healthy and diseased states. Yet, improvements are needed to boast their safety and clinical effectiveness. For instance, potent drugs generate unwanted and potentially damaging side effects as well as producing toxicity in otherwise healthy organs and tissues. Normal physiological functions of tissues and organs can also be damaged when there is an excessive concentration of any therapeutic biomolecules distributed to the incorrect and disease-free site.

These problems can only be addressed through accurate targeting of proteins, minerals, and pharmaceutics into specific tissues and cells delivered using small and highly mobile transplantation units with efficacy equivalent to lentivirus mediated gene-to-cell transfer. In general, the capsule is one of the most effective modules used in targeted drug delivery. Typically, they have dimensions ranging between 100 nm and 10 μm. For tissue modulation and high packing efficiencies for tissue repair, a capsule that is spherical in shape possesses the greatest encapsulation efficacies with corresponding surface areas as well as high capacities for therapeutic ingredients.

Molecules essential to the guidance and regulation of bone morphogenesis and in particular the events associated with mineral deposition and metabolism can also be discovered in the earliest marine organisms as they represent the first molecular components established for calcification, morphogenesis and wound healing. It appears that bone morphogenic protein (BMP) molecules, the main cluster of bone growth factors for human bone morphogenesis, are secreted by endodermal cells into the developing skeleton. In addition, signaling proteins such as transforming growth factor (TGF) and Wnt, prime targets in bone therapeutics, are present during the early stages of marine sponge development. Likewise, ready-made organic and inorganic marine skeletons possess a habitat suitable for the proliferation of additional mesenchymal stem cell populations and promoting clinically acceptable bone formation.

To engineer bone tissues within a culture dish, the utilization of recombinant matrix and growth proteins are vital as they assist in speeding up the growth of cultivated tissues into quantities that is acceptable or sufficient for clinical applica-

tions. The skeletal organic matrices of calcifying marine invertebrates provide an unexplored source that can be potentially used in the extraction of growth-inducing proteins. They have the advantage of being ready-made and retain the native state of the original protein.

Examination techniques such as cell assays, chromatography and proteomic studies can be used to identify and evaluate proteins that can possibly be used in bone repair regardless of whether they are derived from cultivated tissues or extracted from marine skeletons. Given the evidence supporting bone matrix protein analogues in marine invertebrates at the moment along with the techniques established in the retrieval and production of proteins, there is an undisputable possibility that they can potentially be used to regenerate living bone in a clinical environment.

Significant evidence reveals that skeleton building BMP-2/4 and TGF-β can be found within various marine invertebrates such as corals. The latest innovations in long-term marine invertebrate cell cultivation in addition to the best practice maricultural can be implemented to ensure that these proteins are produced in a sustainable manner and that the supply is constant. This approach also ensures that coral reef habitats are not damaged during the collection of specimens.

Tissue engineering frameworks or scaffolds that are intended to imitate and mimic their native extracellular matrix counterparts will possess the best possibility of clinical acceptance and success. The creation of bioceramics that duplicate the inorganic nanocomponents of human bone and organizing these nanocomponents into a hierarchical three-dimensional (3-D) structure is required to translate this scaffold into bone tissue. A variation of this approach is to synthesize and deposit biomimetic nanostructured coatings onto existing implant materials and structures. Coatings and materials derived from sol-gel method can provide nanometric building blocks that mimic the components and structures similar to those observed in biological inorganic matter. Another biomimetic approach it to directly seize or capture already existing structures from natural sources and enhances their function so that they can be utilized to address specific clinical problems or challenges.

There are abundant sources of marine materials and structures as well as techniques that have undergone an evolution that resulted in new and different applications and functions to their original intended purpose. One of the simplest strategies is to select a pre-designed, pre-formed structure such as unique marine structures and to modify it in such a manner that the resultant product can be utilized for any new application in the future [1]. Furthermore, we can study nature and attempt to accurately replicate and reinventing vital components in the laboratory. In addition, we endeavor to learn more from nature the principle of low energy usage in the construction process, importance of structural organization and implementation of transformative self-assembly and non-equilibrium chemistry.

Throughout the past two decades, coral has been recognized as the one of the most successful natural skeletons that has been applied in the clinic. However, corals lack high compressive strength despite its bone emulating properties and in particular its morphology at different scales. Consequently, this limits their use in load bearing applications unless it is fixated with internal devices.

This chapter reviews the current bioceramics and natural marine structures including their structure, morphology, and applications in regenerative medicine, bone grafts, and drug delivery. In addition, the extraction of biological materials such as proteins from marine materials will also be discussed. An example of this specific biomimicry is provided by filtering the microskeleton of Foraminifera and coralline microspheres. New selected strategies based on our research as well as the works of others concerning the engineering of new bone tissues based on biomimicry will be also examined.

1.2 History and Classification of Bioceramics

Bioceramics were utilized as implants since the start of the 1970s to perform a single and biologically inert role. The inadequacies of these synthetic bioceramics as replacements for human tissues were emphasized with the growing awareness that human tissues and cells conduct many other essential metabolic and regulatory functions.

Since then the requirements of bioceramics have changed to providing a more positive interaction with the host from just essentially sustaining a physical function without causing a host response. This has been accompanied by increasing demands on medical devices that they not only improve the quality of life but also extend its duration. Above all, bioceramics may potentially be employed as body interactive materials intended to assist in the healing process or to promote the regeneration of tissues and therefore restore physiological functions.

Basically, a biomaterial is a non-drug material that is ideal to be incorporated into systems which replaces or enhances the role of human organs or tissues. Artificial devices a hundred years ago were manufactured and developed from materials such as wood and gold to a point where they potentially could replace different parts of the human body. Such materials are capable of causing little or no adverse reactions while being in contact with tissues and fluids of the body for an extended period of time.

The reaction of human tissues toward synthetic implants can be of several different ways once these materials are exposed to the body. The reaction observed at the implant surfaces will determine the nature of tissue interaction at a nanoscale level. Consequently, three definitions have been derived to describe the tissue responses of a biomaterial: bioactive, bioresorbable, and bioinert [2].

For any material, if it is classified as bioactive signifies that there are interactions between the material such as hydroxyapatite (HAp) and the surrounding bone and even with soft tissues in some cases once it is implanted into the human body. Bioresorbable for a material such as tri-calcium phosphate (TCP) implies that it will begin to dissolve or be resorbed and slowly replaced by developing tissues such as bone once it is placed inside the human body. Lastly, bioinert denotes that a material will have minimal interaction with its surrounding tissues once inserted into the body.

Examples of bioinert materials include stainless steel, titanium, alumina, partially stabilized zirconia, and ultra-high molecular weight polyethylene.

For any implant, their clinical successes are determined by factors such as biofunctionality, biocompatibility, the experience of the surgeon, the health conditions of the patients, the design of the implant and the interactions at the tissue-implant interface.

Over the last three decades, enhancements in interfacial bonding by nanoscale interactions based on biomimetics have been of great interests to many researchers worldwide. The process of biomimetics is based on the idea that information is processed and stored by any biological system at the molecular level. At the moment, this notion has been expanded to the development of nanocomposites for tissue engineering such as scaffolds for bone regeneration and biomedical devices, and a number of companies are at the early stages of commercializing new-generation implants modified at a nanoscale level intended for applications in soft and hard tissue engineering and for applications in ocular, orthopedic, and maxillofacial surgery [3–6]. Furthermore, numerous research teams have described the synthesis of novel bone nanocomposites composed of hydroxyapatite and gelatin, collagen, or chondroitin sulfate using a self-assembly approach.

1.3 Productions of Bioceramics and Nanobioceramics

A nanostructured material can be defined as a material that is composed of complex structures and dimensions that fall within the limits of 1–1000 nm. During the last thirty years or so, a vast development of nanotechnology has been witnessed because of this size in the areas of materials engineering and science. It is extremely vital to choose the most suitable technique in the synthesis of nanomaterials and nanocomposites with preferred properties or a combination of different properties. This is due to the fact that the properties and microstructure of nanostructured materials are governed by their structure and chemistry as well as how they were synthesized and processed.

In the fabrication of advanced ceramics, the most widely used method includes wet chemical processing approaches (for example sol-gel and co-precipitation) and pressing, all of which have been employed to synthesize nanoparticles, nanocoatings, and nanostructured solid blocks and shapes. Pressing, in modern ceramics technology, is achieved by placing the ceramic powder into a die and pressure is applied causing compaction. Similarly, high-density bioceramics are commonly produced using hot pressing and hot isostatic pressing. Using hot pressing, it is relatively easy to manufacture non-uniform components as well as flat plates or blocks. On the other hand, the smaller grain structures and higher densities required by bioceramics can be satisfactorily produced using hot isostatic pressing where heat and pressure are simultaneously applied from every direction using a pressurized gas such as argon or helium.

In addition to pressing, the unique process of sol-gel can also be applied to manufacture ceramics of various forms such as coatings, platelets, fibers, monoliths, and powders with identical composition simply by changing the viscosity, chemistry, and other features of a given solution. There are a number of advantages associated with the sol-gel method including the manufacture of a pure, stoichiometric and homogeneous product due to the mixing is being carried out at a molecular level, and this high purity can be maintained as grinding can be avoided. Furthermore, a reduction in the firing temperature can be achieved owing to its small particle sizes with high surface areas.

The sol-gel technique has the capability of producing uniform fine-grained nanostructures using either the aqueous-based or alkoxide chemical routes. Using the sol-gel approach to synthesize coatings have an additional benefit as the process only requires a small amount of precursor materials, resulting in their costs being relatively unimportant. Depending on the chemistry, shrinkage is considered relatively uniform perpendicular to the substrate for multi-layered coatings and the coatings can be dried rapidly without cracking. In spite of this, shrinkage is an important factor during the production of ceramic monoliths [3–6].

There is a potential to expand the design and production of new nanomaterials that are beneficial for medical applications using the combination of the unique ceramic production techniques previously discussed and advancements in new enabling technologies such as surface modification techniques, 3-D printing that utilizes both liquid initiated and solid powders, and micro- and nanoscale and biomimetic (bioinspired) fabrications at an unparalleled rate.

At present, the focus is on the manufacture of novel nanoceramics that are applicable for a wide range of functions such as cancer treatment, tissue engineering and regeneration with increased bioactivity, viral and bacterial infection treatment, materials used in minimally invasion surgery, deliveries of gene, drug, and oxygen to damaged tissues, and implantable medical devices that has been surface modifies to achieve improved hard and soft tissue attachments.

1.3.1 Nanocomposites

A nanocomposite can be described as a heterogeneous mixture of two or more materials where at least one of those material should be a nanomaterial. Through the use and assistance of a secondary substitution material, it is feasible to engineer the mechanical properties of the composite (i.e. Young's modulus) to match those of natural bone through the use of the composite approach. At present, one such example is a composite composed of hydroxyapatite and polymeric material, which have been shown to possess a Young's modulus value similar to that of bone tissue.

Nanocomposite can be manufactured by either mixing physically or through the introduction of a new constituent into an already existing nanomaterial and this enables the properties of the nanostructured material to be altered and may even offer new functions or applications. For instance, it has been reported that certain

biomolecules or biopolymers such as poly(lactic acid) (PLA), poly(lactic-*co*-glycolic acid) (PLGA), polyamide, collagen, silk fibrin, chitosan, and alginate have been amalgamated with nano-hydroxyapatite systems.

Another type of nanocomposite is the gel system which has been developed for biomedical applications. With this system, a gel (essentially a three-dimensional network immersed in a fluid) is used to entrap or encapsulate nanomaterials. This results in improvements in the properties of the nanomaterials and can be adapted to meet the specific requirements of individual biomedical prosthetics or devices. An example of a gel system which can be utilized as a drug delivery vehicle is a nanogel. In essence, a nanogel is a nanosized flexible hydrophilic polymer gel and one of the key advantages with such system is that they permit for a high "payload" of macromolecules of up to 50 wt%, a value which normally cannot be approached with conventional nanodrug carriers [7, 8]. Through ionic interactions, these nanogels can spontaneously entrap and bind any kind of negatively charged oligonucleotide drugs. Additionally, an innovative intracellular biosensor has been developed by encapsulating indicator dyes into an acrylamide hydrogel [9, 10]. Moreover, an enzyme-based biosensor was developed using a carbon nanotube aqueous gel as they have been suggested to be an enzyme-friendly platform [11].

1.4 Liposome-Based Delivery Vehicles

It can be said that lipids and bioceramics complement each other. In nature, lipid vesicles are the primary template for controlled biomineralization into marine shells, teeth and bones. Liposomes are synthetic vehicles that can be used in conjunction with proteins and drugs to treat diseases, foreign body wounds and cancer [12]. They can effectively encapsulate and immobilize a wide-range of genes and drugs that are different in size and structure. One of the biggest advantages of liposome is that the hydrogen ion concentration, pH, as well as other ionic concentration can be controlled without affecting the core of the liposome.

In addition, liposomes can also offer protection for encapsulated biological materials such as peptides from being degraded and damaged. Furthermore, they possess higher loading capabilities in comparison to microemulsion, in particular when it comes to water-soluble additives. Stimulations for liposomes is gathered from cells and its intimate similarity to cell boundaries and delineated sacs. In general, single and multilayered spherical bilayer vesicles with thicknesses between 40 nm and 50 μm are synthesized by mixing amphipathic lipids in a polar solvent. Liquid-crystals created by lipids generate highly-ordered structures that can produce three-dimensional bicontinuous cubic organizations and they showed promise as sustained delivery vehicles for peptides and proteins [13].

For any man-made or synthetic delivery systems, they lack the presence of biorecognition molecules which allows specific targeting in cancer and gene therapies to occur unlike vesicles and cells. The addition of a lipid layer to those man-made replicates can enhance their functions. The deposition of a chitosan coating can also

result in such improvements, for instance a reduction in leakage of encapsulated substances as well as an increase in the stability of the delivery system. One important method of mobilizing and directing liposome-based vehicles to a pre-selected destination is to use selective liposome targeting to cell-surface receptors. At the moment, the most effective strategy of solving this problem is through the use of recombinant immunoglobulins. The combination of liposomes and a synthetic polyethylene glycol (PEG) can produce a composite delivery system with enhanced capabilities such as higher targeting potentials.

It has been documented that the use of liposomes has been reduced due to factors such as low encapsulation efficiency and inadequate storage capacity [14]. Liposome-based structures with greater stability and organizations can be produced using crystalline materials with relative ease. Drug encapsulation and elution properties can be controlled and regulated by altering the molecular structure.

1.5 Bioceramic-Based Delivery Vehicles

As previously mentioned, bioceramics represent an important class of biomaterials designed for the tissue engineering of bone, cartilage, and teeth. Biominerals, despite their characteristic crystallographic structure, can be remodeled through specialized biological regulations and controls of mineral deposition on organic membranes as well as within and between cells into an intricate three-dimensional morphologies and richly diverse collection of curved shapes [15–17]. In comparison to mineral crystals created from the laws of physics, a number of the morphologies which are produced by biology are more architecturally complex. On the other hand, silica in general precipitates as spherical colloidal particles, while unicellular organisms can manipulate silica minerals into “lace-like” structures.

Due to their bioresorption capacities and biocompatibility, hard mineralized materials and their derived structures are ideal as substitutes for calcified bone and joint tissues. In terms of bone replacement surgery, the most promising materials that can be applied as a drug delivery system in calcified tissues are biphasic calcium phosphate bone substitutes derived from a mixture of hydroxyapatite and β-tricalcium phosphate [6]. Studies are being carried out to enhance their biological activity using a number of different means, as these minerals by themselves are biologically inert.

In terms of bone repair and reconstruction that utilizes biomimetic drug delivery vehicles, an issue that has become increasingly vital and essential is the combined release of multiple biological molecules and therapeutic drugs from the same system. For example, the opinion concerning the procedure being used in the treatment of osteoarthritis is to achieve a co-operative balance between bone promoting and resorbing drugs and antibiotics. This is due to the fact that there is always a constant and recurrent threat of bacterial infection arising from the highly invasive nature of bone surgery [18, 19].

This belief was applied during the synthesis of biomimetic nanoapatite crystals engineered to release alendronate and anti-cancer and/or anti-metastatic drugs in a

combined manner via the controlled desorption on the crystal surface [20]. The rate of release was governed by the use of either plate-shaped or needle-shaped crystals with various surface areas and charges. The theories and concepts provided by biomimetic materials chemistry has generated a number of key benefits that can be applied in the regeneration of calcified tissues [17, 21].

1.5.1 Nano-hydroxyapatite Powders for Medical Applications

More accurately, bone mineral consists of nanoplatelets rather than nanocrystals initially described as hydroxyapatite, which bears the resemblance to the mineral dahllite. Presently, a consensus has been reached for a description which is more appropriate to describe bone apatite. They are now termed carbonate hydroxyapatite with a chemical formula similar to $(Ca,Mg,Na)_{10}(PO_4CO_3)_6(OH)_2$. The composition of commercially available carbonate hydroxyapatite is similar to that of bone mineral apatite.

Nanotechnology has created innovative methods for synthesizing man-made bone-like nanopowders. Nanopowders and nanoparticles of hydroxyapatite have created new opportunities in the development of nanocomposites for dental and orthopedic applications. They provide excellent bioactivity arising from their extremely high surface area for integration into bone [3–6]. Nanoplatelets and nanopowders of bone-like hydroxyapatite can be produced using a wide variety of synthesis techniques. One approach in particular that showed great promise in the synthesis of bone-like hydroxyapatite is the sol-gel approach. It should be mentioned that monophasic sol-gel hydroxyapatite coatings and powders are more difficult to synthesize despite that fact that previously published works revealed biphasic hydroxyapatite could be produced with relative ease.

At present, nanocomposites consist of hydroxyapatite macro- and nanoparticles and organic and/or biogenic materials such as synthetic peptides, growth factors, and collagen are being produced by a number of companies and research groups. The mechanical properties of the nanocomposite are enhanced through the combined use of macro and nanoparticles as this cannot be achieved simply by using nanoparticles. Increases in bioactivity and mechanical properties have been documented in dental and orthopedic applications with some of the nanocomposite materials as dental fillings and bone cements [22].

The production of porous hydroxyapatite scaffolds has been previously reported using two different approaches based on the manipulation of the hydroxyapatite slurries [16]. The first approach involves the infiltration of a polymeric sponge into the slurry until the inner walls of the polymer are completely covered by hydroxyapatite powders. The scaffold is subsequently fired to remove the polymeric sponge and the resultant ceramic skeleton is strengthened due to the sintering effect at high temperature. The second method involves the utilization of computer-driven rapid prototyping techniques such as robocasting to produce ceramic components with complex shapes and anisotropic microstructures. The process involves the extrusion

of a ceramic ink through a thin nozzle to construct a component one layer at a time following a computer-generated design. Sintering in air at temperatures ranging from 1100 to 1200 °C produces a dense ceramic with narrow grain-size distribution. Both methods are said to be capable of synthesizing ceramic scaffolds with suitable pore sizes to promote bone ingrowth [23].

In another study, a powder mixture composed of biodegradable fillers (β-tricalcium phosphate) and a reactive component (tetracalcium phosphate) was printed using an aqueous citric acid solution [24]. In order to significantly improve the mechanical properties of the printed components, two post-processing procedures, namely a sintering and a polymer infiltration process, were utilized. Samples of various shapes and sizes were printed to examine the feasibility of the developed three-dimensional printing process using a powder-binder system. Initial investigations including in vitro cytocompatibility examination revealed this innovative printing system could be an effective technique in the manufacture of patient-specific ceramic scaffolds and substitutes for bone-tissue engineering.

A powder-binder system was also applied in another study to examine the possibility of creating ordered tubular structures with open porosity using microextrusion free-forming technique [25]. The mixture consists of fine hydroxyapatite powder suspended in isopropyl alcohol with a polyvinyl butyral binder. Tubular lattice scaffolds were produced and sintered at 1250 °C to create a ceramic structure that could potentially be utilized as a bone scaffold capable of encapsulating and releasing growth promoters in a controlled manner.

1.5.2 Calcite and Calcium Phosphate

Highly accessible collections of structural designs are provided by the rich taxonomic assortment of intricate calcite structures throughout the lower orders of the animal kingdom. A number of these structures will offer application for which they had not been originally designed, for instance the reticulated filtration system makes an ideal structure for drug entrapment and delivery by chance.

Presently, natural invertebrate skeletons are almost impossible to replicate using artificial means even though how these structures are synthesized at a molecular level in addition to how they are intrinsically assembled is well-known. As an alternative, chemistry that imitates simple forming phases during the morphogenesis of shell structures has created materials and constructs with similar detailed morphology and function.

A network of submicron and nanoscale interconnected channels and pores are found within naturally occurring for aminiera shells. These networks also provide additional paths for the fluids to flow and could potentially enhance the capability of the shells to accept metabolites, waste products, and growth medium.

The differentiation of osteoprogenitors into cartilage tissues and osteoid can be accelerated once it is cultivated along with structures that mimic the morphology of calcite shells of microscopic planktons synthesized using an analogous process [26].

Furthermore, it was discovered that growth factors could be entrapped between and inside the crystal plates during the assembly of microsphere, and these growth factors are released into the surrounding environment as the calcite is slowly dissolved. In addition, in vivo regeneration of mature mineralized bone and neocartilage is possible by combining these bone conductive microporous spheres with human allograft and human bone marrow stromal cells, the precursors to osteoblasts [27].

Bio-templates are created through the specific arrangements of structural elements such as channels, pores, and struts. These templates can then be utilized to arrange and organize various cell types into anatomically coherent and accurate functional tissues. For instance, the estimated 70,000 different species of corals can provide sufficient structural diversity to match the varied textures found in human bone.

A number of different and unique filtration architectures have been identified from certain species of tropical coral sands. Their unique design, which is composed of macro- and microscopic pores, can be used to control the rate of drug release. More importantly, these coral sands can be completely converted into a variety of soluble calcium phosphates. As mentioned previously, it is extremely difficult and even impossible to replicate these small pores synthetically. As a result, such coral sand makes a novel and indispensable slow drug delivery vehicle. In addition, other pharmaceutic/therapeutic drugs and biological substances such as antibiotics, bone morphogenetic proteins, and stem cells can be incorporated within these structures for any imminent tissue engineering applications.

The unicellular organisms that created these microscopic shells could potentially be cultured and reproduced with high precision inside fermentation containers. This also implies that we are not restricted by any structural designs governed by factors such as the environment and evolution. Accordingly, it is possible for humanity to harness the highly efficient production methods offered by nature for applications such as tissue engineering and drug delivery [28]. By regulating the culturing conditions, growth patterns can be controlled and ultimately the structures being synthesized as demonstrated in a study by Townlet et al. [29].

At the moment, the conversion of coral sand particles that have distinctive filtration structures such as *Schlumbergera floresianus* and *Baculogypsina sphaerulata* into calcium phosphates with controlled solubilities and encapsulating these sand particles with a variety of drugs and biological materials for slow dissolution and targeted delivery intended for applications in tissue repair and regeneration is a key area of research being pursued [4, 6, 19].

Conversely, marine-derived hydrogels have been proven extremely adaptable in experimental biomedicine as a delivery vehicle for growth factors, drugs and genes [30, 31]. Naturally occurring polysaccharides are highly biocompatible with a LD50 equal to table salt [31] as well as being bioresorbable. Alginate polysaccharides derived from chitosan, seaweed, and crab shells are one of the most adaptable substances in use for applications in biomedicine such as gene and drug delivery and tissue engineering [32–35].

Despite their advantages, it is essential for polysaccharides to undergo physical and chemical modifications so that their functions within the human body can be maximized. By far the most useful system for therapeutic intracellular delivery is

chitosan nanoparticles. These nanoparticles are labeled as smart delivery vehicles due to the fact that chitosan is extremely responsive to changes in temperature and environment pH (within a limited range). In addition to polysaccharides, alginates are equally important as versatile vehicles that can be used to encapsulate and deliver genes and proteins in a sustained manner by virtue of their chemical structure; for example, gelling can be the result of changes in the pH level or through the actions of ionic substitutions [36, 37].

1.5.3 Mineral-Coated Polysaccharide Microspheres and Nanospheres

A technique that has been widely applied to increase the number of functions that any biomaterial can perform is the creation of a composite material, through either amalgamation or the deposition of a coating on its surface with another biocompatible material. This necessity arose from the realization that a single biomaterial often does not acquire enough functional properties for a specific application [38]. The resultant composite materials permit the combination of various functional characteristics.

A simplistic technique of adding multiple coatings to an implant is through the use of layer-by-layer assembly [39, 40]. There are a number of advantages associated with this manufacturing process such as the ability to construct intricate hierarchical components from molecular-based units. In addition, this technique can be carried out with relative ease on three-dimensional and flat substrates and an array of substrates can be used.

In comparison to structures at the microscale, numerous unique advantages are offered by nanostructures and some of the most important and distinct features include their ability to load pharmaceutics in a more efficient manner and their increased likelihood of penetrating into cells and passes directly through basement membranes. A common layer-by-layer manufacturing strategy being developed at the moment is centered in the electrostatic association between highly-charged polysaccharides such as highly deacetylated chitosan to produce different composites [41].

The development of better tissue engineering scaffolds that contributes to the natural processes of regeneration through increasing the quantity of natural bio-responsive molecular associations are clearly needed at the present moment. These regenerative processes are extremely dynamic and delicate in space and time. It has been theorized that bio-adhesive building blocks and modules can be produced from unique polysaccharide assemblies combined with functional biomolecules responsible for cell-cell and cell-matrix interactions for active involvement in the re-assembly of cell-mediated extracellular matrix, which are guided and instructed by the cells themselves (both host cells and cells that are being introduced). Moreover, the re-engineering of those bio-adhesive modules will permit the release of soluble proliferation and differentiation elements and to provide support for the formation of tissues within the newly cell-assembled polysaccharide matrices.

1.5.3.1 Production of Tissue Assembly Modules

The creation of tissues that are not only anatomically accurate but also identical in terms of biology and function is the central objective of all tissue engineers. Normally, a suitable environment that allows the organization of cells into functional tissues through self-assembly is created by the engineer [42]. This approach, on the other hand, does not always generate functional tissues with prominent quality. Consequently, the presence of a biological blueprint is essential in the design of the architecture and in organizing the vascular arrangements. One such method that can be used to resolve this issue is to construct building blocks of tissues and guiding the organized assembly into a product that has a high degree of accuracy from an anatomical perspective. The assembly process may be encouraged by factors such as chemical bonding, receptor-ligand binding, or physical forces.

Another approach that displays high potential is the utilization of interlocking elements that have a tendency to amass into complex structures as the procedures that determine how these elements are constructed are written into them, resulting in the production of structures that are highly functional and organized.

There are universal and fundamental design procedures that regulate the construction of biological materials at various dimensions, i.e. molecular and nanoscale [43]. Examples of building blocks that shows promise due to their ability to form specific associations and are high functional include microtubules, de-oxyribo nucleic acid strands, and peptide amphiphiles [44]. The manufacture of these components synthetically inside a laboratory can enhance their ability to control biological outcomes.

A number of functional applications are provided by microtubules including a fixture point for intracellular components, transportation link for moving materials, and most importantly providing structural integrity for cells. Newly constructed microtubules can be re-arranged from simple tubulin monomers by means of chemical stimulation followed by the application of a direct mechanical force [45, 46]. Sufficient amounts of energy are provided by this combined action to produce the bonds between the tubulin monomers. Conversely, the combination of mechanical interaction and chemical stimulation can also be used to dismantle this bond. In the same way as tubulin, de-oxyribose nuclei acids can also be used in the engineering of new structural frameworks with high integrity as they are categorized as self-assembly building blocks.

Significant amount of progress has been made on the synthesis of new biomaterials using molecular-engineered proteins and peptides [44]. The creation of new frameworks together with the supervision and involvement of living cells applied to the re-development of tissues is an attractive objective to be pursued as extracellular framework with desired functional and structural properties can be regenerated by accessing the genetic programming of the cell. A relationship exists between every cells and simplified building blocks and modules that represents an element of the extracellular matrix displaying certain molecular motifs, for example sulphation codes on glycosaminoglycans (GAG's) [47]. These components are initially synthesized followed by the secretion of cells they are related to.

A hypothesis based on this phenomenon worthy of exploring relates to the deposition of biorecognition ligand molecules suitable for the integrin protein discovered on the surface of the selected cell to facilitate the possible establishment of new environment once contact is made between individual cells. In comparison to peptide-free surfaces, higher cell recruitment can be theoretically achieved by binding the polysaccharide capsules with a universal cell adhesion tripeptide such as arg-gly-asp-RGD after 24 h of incubation. However, it is vital to determine the size, length, surface density, and distribution pattern of peptides as these factors are known to have profound effects on cell migration and adhesion [48, 49]. In addition, a study has revealed the stimulation of myoblast cell phenotype can be intensified if the densities of RGD attached to alginate is in the range of 1–100 fmol/cm^2 [36].

1.5.3.2 Multi-layered Mineral-Coated Polysaccharide Spheres

Polyelectrolytes have been utilized to govern and control the release profile of the encapsulated substance during the manufacturing of the vehicle used in controlled drug delivery [40]. A number of advantages are offered by polyelectrolytes including their ability to assemble rapidly and effortlessly in mild conditions and with high functional efficacy [39].

Multiple concentric layers of biomaterials can be deposited onto polysaccharide spheres by electrostatic attraction between individual oppositely charged substrate layers. Entrapments of drugs and growth factors inside the water-filled space between membranes can be achieved using hydrogels consisting of multi-layered membrane, similar to the construction of an onion. More importantly, the intended application will determine the number of layers used to produce the hydrogel [50]. Alginate, β-chitin [32, 51–53], hyaluronate [54], chitosan [41, 55] have been identified as potential candidates in the production of multi-layered hydrogels with the aim of compartmentalizing a variety of therapeutic and/or pharmaceutic substances at different concentrations within the series of concentric shells. Furthermore, the individual layers of the concentric shell can be manufactured with customized properties such as degradation and permeability. Physical entrapments of cells and therapeutic proteins such as TGF-β3, BMP-2, and chito-oligomers for bone tissue regeneration at the interface between two concentric shells have been shown to be viable. Likewise, through the application of carbodiimide chemistry, proteins can be chemically conjugated and adsorbed into the polysaccharide substrate [36, 56, 57]. In addition, an increase in the resistance to mechanical forces and stability of chitosan shells can possibly be achieved through the addition of amorphous calcium phosphate [58].

The layers can be unwrapped one layer at a time towards the core of the concentric capsule and the encapsulated proteins are released during peeling process. This process also ensures the rupture of the entire capsule is belated. The calcium and phosphate ions within the crab shell chitosan and sodium alginate forming solutions are used to create an outer shell encasing the capsule and the concentrations of the ions are used to program and adjust the fracture and swelling rates of this outer shell.

Furthermore, a relationship exists between the thickness of the outer shell and the concentrations of the calcium and phosphate ions as the concentric capsule hardens. A thicker outer shell is produced by increasing the concentrations of the calcium and phosphate ions, and this in turn increases the time needed to rupture the capsule. On the other hand, the shell will become extremely brittle if the concentration of calcium ion exceeds 50 mM and phosphate ions is greater than 300 mM.

Similarly, entrapment of cells between individual layers is also possible, and the creation of concentration gradients of growth factors, proteins, and genes within every individual polysaccharide capsule that is significant physiologically from the exterior to the core is feasible with such assemblies.

1.5.3.3 Biomimetic Cell Assembly Mimics

Tissue regeneration operates alongside drug therapy and discussions have been raised concerning the possibility of harnessing certain cells to replace drug compounds by genetically engineering these cells to release therapeutic proteins in conjunction with local physiology. Furthermore, the same role may also be undertaken by harnessing the functional unit of cells. Cellular assemblies can be considered as functional components surrounded by an ordered arrangement of cells of various types with distinct roles in the reconstruction processes of tissues. The ability to synthesize basic units of tissues followed by their construction into multiple hierarchies and layers in the laboratory will be regarded as highly advantageous. In simple terms, this means that synthetic building blocks are used by the selected cells to create their own unique structures under suitable environments.

Several factors based on human biomechanics, partition chemistry, and biology governs the formation of mineralized tissues and regulates their assembly into defined spaces. The assemblies of cells are limited by borders comprised of impermeable substrates that encompass the mineralization in addition to regulating the chemistry and structure of the fluid-occupied environment [17, 21]. Cells arrange themselves into groups within the bone during the mineralization process. The collaborations of osteoblasts to produce fluid-filled compartments that separates from blood and other mineralized tissue is one of the hypotheses that has been proposed to describe bone growth. Moreover, fluid-filled regions are isolated from already existing mineral wall and organic polymer sheet [59].

The use of modular self-assembly to synthesize novel biomaterials is a process that has been well-established. The design and fabrication of, via biology in real-time, a new category of biomaterials can be achieved using the cell-mediated approach. The re-assembly of the matrix environment of human progenitor cells using synthetic and engineered multi-component biomaterial segments or “building blocks” is an idea that is still progressing. Cell-engineered biomaterials can be re-designed with optimized properties accurately with this approach [60].

Pre-fabricated scaffolds are currently being developed with integrated bio-recognition motifs or ligands by tissue engineers that are regulated by activities that are naturally occurring as well as responses by progenitor cells observed during the

early development ad regeneration of tissues in adults. Simulating the complex time-dependent interactions amongst individual cells and between cells and its matrix has been attempted. For instance, the fabrication of polymeric scaffolds stabilized with cross-links and ligands that can only be destroyed by matrix metalloproteinase-3 (MMP-3) has been achieved and their structure is altered once metalloproteinases are secreted by cells locally [61–63].

This alteration is designed to stimulate matrix events that provide re-enforcements to the regeneration process. The development of cell-independent self-assembling biomaterial structures that continuously simulate their natural counterparts has been the objective amongst different researchers. Improvements in both the quality and rate of tissue regeneration in addition to maximizing biological responses are provided by both techniques.

Microsponges composed of calcium carbonate with dimensions at or below that of a single cell were controlled in past studies by individual cells and organized favorably by co-cultured human bone marrow stromal cells into mono-layered aggregates with various cell densities [26]. The feasibility of coating the external surfaces of polysaccharide spheres with GGGRGD (Gl-gly-gly-Arg-gly-asp) peptides has previously been demonstrated which allows the preferential attachments of cells to the outer surfaces [56]. Furthermore, the amalgamation of crab shell chitosan with RGD tri-peptides in solution was carried out before the deposition and polymerization onto an alginate droplet [30].

The creation of cellular assembly mimics has also been hypothesized, and in essence they are biocomposites consists of human cells and polysaccharide that imitate the conversion process to discrete mineralized modules from mature bone cells that occurred naturally. This transformation signifies the precursors to bone reconstruction [59, 64, 65]. The manufacturing of cellular assembly mimics utilizing polysaccharide modulus as starting materials applied in bone reconstruction is one of the primary capabilities that are being developed. The other is to create cell-recognized matrix elements that can be re-organized into macromolecular-scaled cellular niches.

The transportation of regenerative factors in the correct amount to treatment site in a chronological order has proven to be rather intangible. The difficulty stems from technical issues such as the synchronized release in collaboration with host physiology and the number of biological responses which can occur accordingly [66, 67]. The stability of proteins is limited as they degrade too rapidly. In comparison to the delivery of proteins to cells directly, strategies based on gene correction are intended to enhance the potency and effectiveness of protein synthesis and secretion form the cells [68, 69].

Factors such as selecting the appropriate synthetic biomaterials and lipids and the physical disruption of the cell membrane to permit the infiltration of new and prominent genes and transcription factors determine the success rate of cell-mediated gene therapy that utilizes non-viral transduction agents. Unproductive inefficiencies in gene expression levels and targeting along with inappropriate gene integration are associated with all of the aforementioned processes. A method has been reported in which the transfection efficiencies increased to more than 65% and a toxicity level

is reduced to less than 5% [70]. Furthermore, an increase in transfection efficiencies can be achieved through the use of lipid DNA polycondensates and cationic polymer, while at the same time eliminating issue of lysosome degradation [71, 72].

The release of encapsulated bioactive compounds in synchronization with the body's own biochemistry at dosage levels beneficial for cells in precise orders and for certain periods of time is of vital significance. Such approach will ensure maximum potency and efficacy is provided by the encapsulated compounds.

One of the key challenges concerns the release of individual compounds for extended periods in a slow and sustained manner aimed at permanently restoring the functions of tissues. Two methods can be utilized to control the release rate of the encapsulated substance. The first approach is based on the alteration of the shell of the delivery capsule in which the thickness and composition is adjusted, and in view of that, this technique exploits the theory of diffusion to slow the rate of release of the uploaded content. This approach has been proven effective in slowing down the release of plasmid de-oxyribose nuclei acid.

The second technique is centered on the fabrication of capsules one inside another to produce a nested arrangement [30]. This "host-guest" combination of capsules is somewhat effective in the release of encapsulate into the surrounding medium in a timely order. Under experimental conditions, capsules containing tyrosinase were synthesized and inserted into a vacant host capsule. This process was repeated twice, and all the capsules contain identical volumes of tyrosinase. The volume of enzyme that must diffuse through is increased through this repeated nesting process, and subsequently the release rate to the surrounding environment is decreased.

1.6 Concluding Remarks

Biomimesis is an idea with rising importance and relevance to a diverse range of sciences and technologies such as biology, materials, nanotechnology, and medicine. Gaining deeper insights into how nature works is vital to the research and development of smart materials, structures, and processes with self-actuating, self-stabilizing, and self-assembling properties. Tissue engineers are confronted with challenges related to the manufacturing of scaffolds with numerous functions (these functions often contradict with one another) that must be bio-responsive and evolve to a dynamic host environment in real-time.

The principles of bioinspired approach used in the design and production of tissue engineering scaffolds have been well-documented and emphasized through BioTriz methodology. There are at the moment additional techniques that are centered on nanotechnology and the synthesis of materials and structures using nanoparticles and nanomaterials. Several recent examples are displaying how biomimesis can be used to create innovative functional tissue engineering frameworks with morphologies and structures (both intricate and complex) unachievable using conventional production techniques.

Regenerative medicine is confronted with problems related to a shortage in clinically relevant scaffold designs and biological factors that promote the natural cycle of regeneration. Obtaining a greater understanding into hierarchical design in nature and harnessing the chemical properties of natural structures at all dimensional levels such as macro, micro, and nano will be instrumental in the re-assembly of functional structures that mimics natural skeletal design. We believe in utilizing bio-inspired and nanoscale materials chemistry to achieve this goal. Such knowledge will also enable tissue engineers to synthesize new and advanced bio-structures and materials that are truly patient-ready as well as being capable of responding to the functional demands imposed during the regeneration of native human tissues. Moreover, the internal microenvironments for embedded cells can be modulated to recreate elements of a native extracellular matrix adding an additional biomimetic element to this unique system.

Bioevaluation delivered convincing evidence that these scaffolds may offer clinical success as novel scaffolds or gene/bio-factor delivery vehicles for the engineering of both mineralized and soft human tissues.

In this chapter, we have provided an example of a self-assembling organic scaffold in which spontaneous mineralization of calcium phosphate can occur. This same procedure is comparable to the template-mediated mineralization observed in nature such as the mineralization of eggshells.

There is a rapidly expanding and ongoing diversification of pharmaceutics and therapeutic proteins, carbohydrates, and lipids. This has been stimulated by the increased knowledge of molecular events in both healthy and diseased organs and tissues. On the other hand, improvements are needed in order for these substances to be used safely and effectively under clinical conditions. For example, potent drugs produce unwanted and potentially damaging side effects and toxicity in otherwise healthy tissues and organs. Excessive concentration of any therapeutic biomolecule distributed to the incorrect or diseased-free locations can also degrade normal physiological function. Such problems can only be addressed through accurate targeting of proteins to the specific tissue and cells delivered using small and highly mobile transplantation vehicles with efficiency equivalent to lentivirus-mediated gene-to-cell transfer.

Biomedical engineers are continually motivated to improve and transform therapeutic medical treatments in an attempt to reduce the invasiveness of surgery in addition to the amount of pain, inflammation, and surgery time needed with drug and protein delivery vehicles. However, delivery systems currently utilized are deficient in two critical ways: inaccurate targeting and delivery is not sufficiently well regulated.

Consequently, ongoing research and discovery of new and more clinically acceptable technologies that can remotely arrive at a designated tissue site and deliver drugs, growth factors, or genes continuously in any specific three-dimensional and chronological patterns. Spheres (with low dimensions) can be applied effectively to transport these biological modules and protect them from physiological degradation. Furthermore, they conform to the shape of the cell during transportation.

Universally, transportation capsules with dimensions that fall within 10 μm–100 nm are one of the most effective unit used in targeted delivery. Capsules in the shape of a sphere possess minimal surfaces areas and a volume where a large amount of therapeutic constituents can be encapsulated. Spherical capsules are also known for their high encapsulation efficacies for tissue modulation, and high packing efficiencies for tissue repair.

Applying the lessons learnt from basic versions in the natural world and from functional replicas in human biology could provide unique answers to these challenges. The first and most difficult step is the discovery of biological analogue that is best suited for carrying out the function we want to develop. For instance, in human biology, studying matrix vesicles will provide us valuable insights into how proteins are captured and coated in addition to how these vesicles is able to dock and fused to their target. A second role model for biomimicry is the filtering microskeleton of foraminifera.

We have selectively highlighted biomimicry approaches to produce new devices that may potentially deliver drugs and genes to their intended destination in correct dosages. Biomimicry, in this chapter, involves the selection of suitable analogy from nature that solves similar problems to the one under examination. Often, we noticed that non-human biology provides simpler and more convenient solutions. We have also examined how lipid vesicles can be routinely self-made to mimic their natural counterparts. We have also showed how bioceramic spheres were made synthetically using biomimicry chemistry for capturing and delivering growth factors to osteoprogenitors. Equally and perhaps more advantageous is the direct use of natural skeletons suited to deliver bone promoting drugs and antibiotics in tandem.

In the future, cells may also play a part in the delivery of beneficial therapeutic proteins. In all probability, this might be the best way forward since cells can be genetically programmed to secrete proteins in synchronization with the host. Future work may also encompass ways of detecting the status and position of integrated devices.

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During in his formative years Prof. Ben-Nissan worked on Titanium and its alloys and Magnesium alloy development and casting technologies and their properties for both engineering and medical applications. Over the last four decades, Professor Ben-Nissan has worked and contributed to the biomedical materials, implant design, production and analysis of various advanced ceramics, nano-coated sol-gel developed thin films, coated orthopedic and dental implants, anti-microbial slow drug delivery devices and methods, marine structures for clinical

applications, biomechanics and finite element analysis of medical materials and engineering structures.

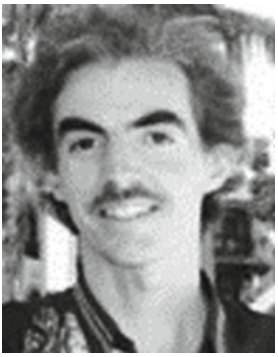
He has successfully developed materials for implant technology such as ceramic knee prosthesis, calcium phosphate based bioactive materials, bone graft production and bio-composites, and conducted research on biomechanics and modelling such as jaw bone, knee and hip joints, reliability and implant design modular zirconia ceramic knee prosthesis, femoral head and taper stresses and artificial ocular implants and bionic eye and recently on 3D printing of bioceramics and metallic implants and anti-microbial multifunctional coatings for drug delivery which are supported by the European Commission and the Australian Academy of Science research grants.

Since year 2000 he has published over 200 fully refereed papers in journals, and a book and 43 book chapters. He edited a book on Calcium phosphates and working on a second one on the use of Marine Structures in the Biomedical field. He is the editor of the Journal of the Australasian Ceramic Society. He was awarded by the Australian Ceramic Society's prestigious award for his contributions to the "Ceramics Research & Development and Education in Australia". For his research on multifunctional nanocoatings he also received "The Future Materials Award".

Andy H. Choi Dr. Andy Choi is an early career researcher who received his Ph.D. from the University of Technology Sydney (UTS) in Australia in 2004 on the use of computer modelling and simulation known as finite element analysis (FEA) to examine the biomechanical behavior of implants installed into a human mandible. After completing his Ph.D., he expanded his research focus from FEA to sol-gel synthesis of multifunctional calcium phosphate nano coatings and nano composite coatings for dental and biomedical applications.

In late 2010, Dr. Choi was successfully awarded the internationally competitive Endeavour Australia Cheung Kong Research Fellowship Award and undertook post-doctoral training at the Faculty of Dentistry of the University of Hong Kong focusing on the application of FEA in dentistry and the development of calcium phosphate nano-bioceramics.

He is served as an associate editor for the Journal of the Australian Ceramic Society and on the editorial boards for a number of dentistry, nanotechnology, and orthopedics journals. To date, Dr. Choi has authored over 50 publications including 3 books and 26 book chapters on calcium phosphate, nano-biomaterial coatings, sol-gel technology, marine structures, drug delivery, tissue engineering, and finite element analysis in nanomedicine and dentistry.



David W. Green Dr. Greem is working at the moment the bioengineering interface between physical, chemical and biological phenomena; He is attempting integration of non-living matter with living matter for manufacture of bioinspired systems. Consequently, to revitalize cells and tissues into novel regenerative therapies. Accordingly, he is guided by biomimetics and bioinspiration philosophies to create new innovations for healthcare. Presently, Dr. Green is focused on biomimetic development of anti-biofilm materials and stem cell niches.

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Sophie Cazalbou Assoc. Prof. Cazalbou's research activities mainly concern the formulation, shaping and characterization of new bioactive biomaterials mainly used as bone substitutes and capable of releasing in vivo active substances such as ions, molecules, and proteins. She is currently interested in developing new minerals and composite biomaterials in supercritical CO_2. This process of "green chemistry" opens new perspectives in the synthesis and development of highly reactive ceramic with controlled architecture. She is working on the following areas: (1) Formulation of biologically active biomaterials (such as coatings, ceramics, cements, composites); (2) Formulation of biomaterials used as delivery systems for active substances (such as antibiotics, anti-inflammatories, growth factors, biologically active ions, bisphosphonates); (3) Influence of microstructure on the properties of transport through the pore space (transport of active species, biological fluids and cells); and (4) Theory of percolation used as pre-formulation element.

Chapter 2
Remarkable Body Architecture of Marine Sponges as Biomimetic Structure for Application in Tissue Engineering

Eva Martins, Miguel S. Rocha, Tiago H. Silva and Rui L. Reis

Abstract Recent advances in the study of marine environment, particularly of marine organisms' architecture and composition, have isolated interesting compounds as proteins, GAG-like polysaccharides and bioactive compounds. These compounds have allowed the development of panoply of biomaterials inspired by morphological characteristics and anatomical structures of the marine species. Besides, the scientific community acknowledges the enormous biotechnological potential in the marine resources that can be a promising effective and efficient alternative to be used in Human health, namely tissue engineering and regenerative medicine, as well as to support the progress in pharmacological, cosmetic, nutraceutical and biomedical fields. Additionally, sustainable ways are being applied to explore these marine resources and address biomimetic approaches, aiming to take the most out of the astonishing marine environment in ecologically compatible ways. Marine sponges are a particular group of organisms feeding these biotechnological developments for human health, both as source of new drugs or inspiration for the development of marine biomaterials. This chapter aims to demonstrate, in a concise and clear way,

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the biotechnological potential of marine sponges used as susceptive bioscaffolds for regenerative medicine and biomedical applications in general.

Keywords Marine sponges · Skeletons · Skeletal elements · Spicules · Collagen · Chitin · Biosilica · Polyphosphates · Biomineralization · Tissue engineering · Biomimetic materials · Biomedical application · Bone · Marine biomaterials · Marine biotechnology

2.1 Introduction

The marine environment provides an abundance of resources and its valuable biodiversity is a potential source of new beneficial products for society, including products for biotechnological and biomedical applications. Surprisingly, marine organisms remain a largely unexplored resource, although a growing interest in this promising field is changing this scenario. Among the marine organisms, marine sponges present a particularly interesting therapeutic potential. These sessile animals classified into the phylum Porifera dating from over 580 million years, developed an important evolution feature by established symbiotic interactions with surrounding microorganisms to protect themselves from pathogenic agents, possessing a broad range of molecules with different effects such as antitumor, antiviral, anti-inflammatory and antibiotic effects [1, 2]. In fact, one-third of the compounds obtained from marine organisms in the last 50 years is obtained from this phylum [2].

In addition to the impressive chemical properties, sponges present outstanding structural features, an inspiration for the emerging area of tissue engineering, an interdisciplinary field mainly focused in the regeneration of functional human tissues by engineering tissue templates mimicking the architecture and functional properties of extracellular matrix (ECM), known as scaffolds, that will support cell growth and differentiation towards net tissue formation. Thus, the development of innovative tissue engineering technologies can benefit greatly from the study of high-performance biostructures and the smart design of marine sponges [3]. Tissue engineering has been studied as a step further to current therapies, corresponding in fact to a change of paradigm, from substitutive medicine to regenerative medicine. Nowadays, the medical procedures for repairing critical injuries that the body could not regenerate by itself are the replacement of the damaged tissue with synthetic prostheses and tissue grafts. However, these clinical practices have numerous complications associated, as potential harmful immunological responses, high medical treatment costs and a limited number of the donor tissues, which prompted the development of tissue engineering procedures [4].

The abovementioned scaffolds have important biological features in tissue engineering strategy, as they maintain a matrix for tissue regeneration in vivo while providing a 3D physical support for cell culture in vitro, thus mimicking more closely the in vivo conditions of the tissue to be replaced than cellular monolayers [5]. However, these scaffolds require specific features, such as adequate biodegradabil-

ity, low immunogenicity and cytotoxicity, suitable pore size (100–600 μm) and pore interconnectivity (porosity > 50 Vol.%) to support cell growth, differentiation and migration [6].

The combination of these demanding properties is hard to achieve and the selection from a vastness of raw materials, ranging from ceramics and polymers to composites, is difficult and present limitations to their use in the preparation of perfect biomaterials. These limitations are being tackled by using natural materials, which provide an interactive surface for cell attachment, growth, and differentiation, while being more biocompatible and biodegradable than synthetic-made materials, as natural-based materials are more similar to the tissue components being mimicked.

Marine sponge skeletons possess noteworthy materials in their composition suitable for tissue engineering such as biosilica, polyphosphate, collagen/spongin and chitin [7, 8]. These are being considered particularly for bone tissue engineering strategies, since the first two inorganic polymers can induce osteogenesis in vitro thus favoring mineralization [9], while the latter two are promising biomaterials for the development of new scaffolds, being a similar and safer alternative than mammalian collagen [10, 11].

Just as previously stated, the developed scaffolds require a suitable structure in order to support cell growth and migration along the whole material network. In this context, inspiration can be taken from nature, as biomimetic approaches can lead to functional and efficient results. Marine sponges present a porous and interconnected architecture and their efficient aquiferous system resemble the trabecular network of the bone tissue [10]. Furthermore, due to the spicules, some sponges have a rigid framework that grants them inherent toughness and stiffness, which are favorable mechanical properties for a bone tissue scaffold [12]. Naturally, the body architecture of sponges is clearly advantageous for bone tissue engineering. However, there are some hurdles to overcome, such as the fact that different sponge species possess different characteristics and compositions, being difficult to achieve reproducibility of the biomaterials and the animal supply must be sustainable and without disruptions [13].

This chapter will describe the state of the art of biomaterials inspired by the unique morphological structure of marine sponges and their potential for tissue engineering, highlighting their morphological organization, skeletal structures as spicules and efficient water-conducting system, as pore interconnected network enabling cell migration and proliferation across all the structure. The sponge skeleton has a simple to complex fibers network constituted by interesting variable biomacromolecules in different sponge species, as spongin and chitin. These biomacromolecules are being used as susceptible bioscaffold for promoting cell attachment, adhesion and proliferation and the latest developments on this route will be also addressed.

2.2 Marine Sponges as Outstanding Biomodel

2.2.1 Phylum Porifera

Sponges constitute the phylum Porifera (Metazoa), being the most primitive of multicellular and filter-feeding animals with an anatomically simple organization. This phylum is formed by mainly sessile metazoans and their aquatic members colonized a wide variety of habitats such as springs, falls, swamps, rivers and reefs but also the depths, from shallow to abyssal environments [14].

The sponges are considered representatives of the primordial multicellular animals and retain a combination of features that qualify as successful animal phylum [15, 16]. These animals have an important ecological role in marine ecosystem but sponges are also a potential source of novel bioactive compounds, synthesized by the interaction of sponges with symbiotic bacteria belonging to different phyla, providing new natural products and therapeutic drugs with perspective to improve the quality of human life [17–19].

In fact, most of the marine natural products in preclinical or clinical trials were obtained from marine invertebrates, predominantly sponges, tunicates, bryozoans or mollusks [20, 21]. Focusing in marine sponges models, sponges are typically difficult to maintain in aquaculture systems: beyond the requirement of large volumes of water that they usually filter, sponges in situation of poor water quality reduce their filtration rates, and consequently they showed a steady state decline in mean length in the few species that are possible to farm yet [22, 23]. Thus, a sustainable use of sponges is being investigated; however, the culture of sponge' cells remains a challenge as results from Muller and colleagues revealed that is harder to maintain a suspension culture of a single cell in laboratory conditions than sponge cells that have an organized tissue-like structure [24].

Commonly the skeleton and spicule structures of the marine sponges are the morphological structures used for taxonomic assignment. The classification into distinguishing taxonomical classes is based on the chemical composition of their skeleton/spicules. According to the Hooper and Van Soest, originally three classes of sponges were considered to their systematic classification [17]. Posteriorly, phylogenic study separated sponge species from Demospongiae class to a new class designed to Homoscleromorpha [25]. Actually, the classification of sponges are still controversial; however, it is majorly accepted the subdivision into four classification classes namely Hexactinellida, Demospongiae, Homoscleromorpha and Calcarea (Fig. 2.1) [26, 27]. The sponge members of each class may exhibit diverse morphologies, physiological and biochemical mechanisms that difficult their precise taxonomic classification.

Currently, the Porifera Phylum has registered 22,487 species subdivided by the four classes of marine sponge (Table 2.1). The most representative number of species is from Demospongiae class with 19,069 species according to World Porifera Database (WPD). Although, only 7394 sponge species were fully validated in Demospongiae class, which represents at least 61% unconfirmed identified sponges. These

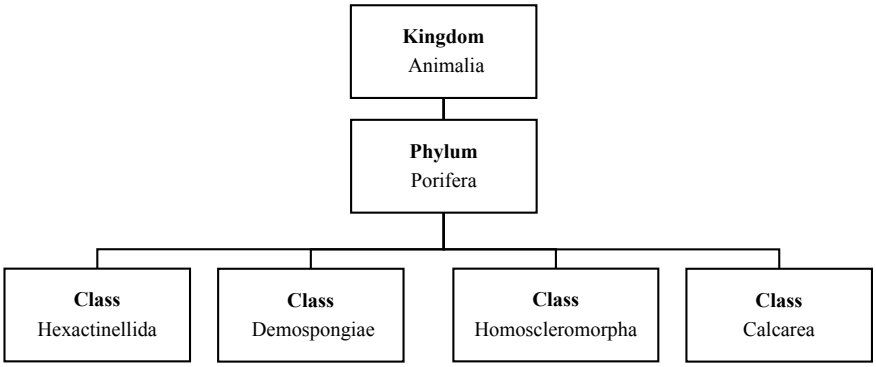

Fig. 2.1 Schematic taxonomic tree of sponges

Table 2.1 Number of all species and accepted marine species from each sponge classes and phylum according to WPD

	All species	Acc. species
Hexactinellida class	1536	633
Demospongiae class	19,069	7394
Homoscleromorpha class	171	109
Calcarea class	1700	740
Porifera phylum	22,487	8877

numbers appoint to the requirement of investigation and further insight in marine sponges' research .

2.2.2 Hierarchical Structures in Marine Sponges

Marine sponges display unique physiological features from nanoscale to the macroscale, as spicules and body networks, organizing in skeletons that are truly hierarchical structures with wide potentialities for various biotechnological purposes.

2.2.2.1 Marine Sponges Astounding Body Architecture Features

Marine sponges are metazoans animals which unique micro-architectural features are being a target of study by the scientific community. Sponges can have their body organized in radially symmetrical or asymmetrical architectures, taking into account their geometric shapes that can be calculate, tubular, arborescent, flabellate, globular or amorphous. In sponges, indeed, their morphological structures can display

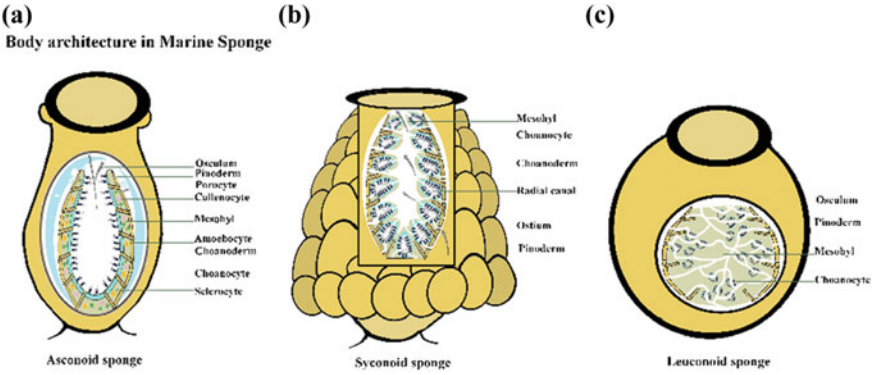

Fig. 2.2 Schematic representation of the types of body architecture and water-canal systems in Asconoid, Syconoid, and Leuconoid sponges

a variety of colors, sizes, cell types and structurally be organized in three distinct layers. The single outer layer of cells designed by the pinacoderm that separates the sponge from the external environment and contains a protein matrix with rove cells; the mesohyl inner cellular layer, and a layer containing the flagellated collar cells, the choanocytes [28]. In filter-feeding animals, the aquiferous system connected by an efficient network of water-conducting channels has an essential function and a simple type of structural organization. Furthermost, three types of sponge structure from the simple to complex regarding to the cells organization and water canal systems may be classified as asconoid, syconoid and leuconoid (Fig. 2.2) [29]. Thus, as previously mentioned, sponges may have branched shapes and distinct anatomical features accomplished by outstanding diverse skeletal structure organizations that enable their classification in different sponges classes (Table 2.2).

2.2.2.2 Skeletal and Spicules Structures Elements

The formation of skeletal features in sponges involves hierarchical mechanisms that secret organic fibers and mineral deposits. Initially, the organic molecules of skeletal structure are produced and then the deposition of inorganic elements occurs, yielding the small skeletal elements, the spicules [7]. Mostly, the sponge skeletons can be made by different elements as spongin (similar to the collagen protein), chitin, calcium carbonate and/or silica depending on the sponge' species that enclose an internal meshwork also formed by spicules elements produced by the scleroblast cells [17].

During the filtration process, these specialized cells sequester silica or calcium from the seawater and continue the biomineralization process for the production of the spicules skeletal elements in sponges. The microstructural diversity of these spicules is a well-connected structure with stylish design and numerous shapes. Moreover, spicules are morphological features present on mesohyl layer in sponges with

Table 2.2 Skeleton features are distinct in the four marine sponge classes of adapted from [17, 24, 30]

Hexactinellida	Demospongiae	Homoscleromorpha	Calcarea
Skeletons are formed by amorphous, hydrated and non-crystalline silica		Skeleton has tetraxonic siliceous spicules without a subdivision in mega- and microscleres. The presence of spicules is a low number or even absent	Skeleton has calcareous spicules formed chiefly by calcium carbonate in crystalline forms (calcite, aragonite)
The skeletal elements are composed by siliceous spicules could be megascleres lengths. The class is known as a glass sponges due to their similar glass structure	Skeletal architecture is very diverse, made from siliceous spicules and protein (spongin) fibers or a combination of organic and inorganic elements		

sizes from micrometers to centimeters (microscleres or megascleres) length [24, 28]. The spicule may precipitate from silicate salts creating the siliceous spicules or the spicules may precipitate from calcium and carbonate ions producing the calcareous spicules. Interestingly, spicules can have a highly variety of shapes for each sponge species that enables their identification [28]. The unique mechanical properties of biological structures as bone and sponge skeletons are a result of their competent design architectures. In *Euplectella* sp., within hexactinellid sponges also known as glass sponges, their skeletal elements comprise tree ring-like layers, which confer extraordinary biological properties [31]. Remarkably, the skeletons from *Euplectella* sp. have hierarchically arranged well-defined glass skeletons with an exceptional mechanical rigidity and stability properties, which is surely an interesting framework for fabrication of tissue engineering scaffolds and for various applications [32].

2.2.3 Components of Marine Sponges Skeletons

2.2.3.1 Biosilica

Sponges have the ability to synthesize enzymatically silicon dioxide (SiO_2), also known as biogenously formed polymeric silica (biosilica), to produce their siliceous skeleton and spicules, strong and flexible marine sponge skeletal elements, with highly complex structures and different levels of hierarchy from the nano to macro levels.

This biosilicification process occurs under their natural physiological conditions in aqueous media at near neutral pH and ambient temperatures, being biologically regulated. The biosilica organized deposition process is guided by a collagen organic matrix. Other biosilicifying organisms include diatoms (micro-alga), radiolarian and plants, although only sponges have the ability to polymerize silica generating large spicules. The silica-forming activity is usually high in sponge tissues, as exemplified by the demosponge *E. fluviatilis* which has an impressive spicule growth rate of about 1–10 $\mu m/h^{-1}$ [33]. The silica element is the predominant inorganic component of the spicules in demosponges, but the incorporation of other elements, such as Na^+ and K^+, is possible to occur in the spicules [34]. In marine sponges, biosilica has the functions of serving as a structural support, providing protection from predators and acting as advanced sensors (e.g. biosilica as optical fibers) [32, 35].

The silicatein enzyme has proven to be very versatile, being involved in biomineralization and responsible for the formation of the siliceous spicules. Interestingly, the growth process of the spicules presents similarities to the bone formation mechanism: the central core rod of the spicule is first synthesized by a silicatein fiber, becoming surrounded by an organic silicatein layer, which will synthesize the second siliceous layer and so forth [36]. In both mechanisms, there is interplay of various factors that are functionally defined in a spatio-temporal context, so the studies on biomineralization must consider a series of well-tuned molecular pathways and interactions. Therefore, the elucidation of the biosilicification mechanism will allow the development of new materials and technologies for a broad range of biotechnological applications with significant impact, including the understanding of the formation of complex skeletal structures.

Regarding the calcareous sponges, the possible interaction of the carbonic anhydrase enzyme family in the formation of calcium carbonates to build calcitic spicules of the calcareous skeletons was described [30]. Interestingly, it was reported a highly hydrated amorphous network of silica in the demospongiae sponge *Tethya aurantia* [37] and a skeletal structure composed of elaborate cylindrical structures with six hierarchical levels was described in the hexactinellid sponge *Euplectella marshalli* [32].

Taking into consideration the biomimetic experiments inspired in marine organisms using, for example, recombinant carbonic anhydrase to form amorphous pat-like particles, it is evident that there was an improvement regarding our knowledge of the intricately basic functions and the chemistry of silica in the biomineralization process, which enables researchers to synthesize new materials [38, 39].

Cleary, biosilica and silica-based biomaterials are excellent biocompatible materials with enormous potential for biomedical applications in the areas of sensors, coatings, hybrid materials, biocatalysis and drug delivery, having particularly beneficial effects on bone and cartilage healing due to their capacity to increase mineralization (formation of mineralized calcium phosphate nodules or hydroxyapatite) [40]. For this reason, they are proper bone filling materials used to develop tissue-engineering scaffolds.

2.2.3.2 Polyphosphates

Polyphosphates (polyP) are inorganic polymers widely present in prokaryote and eukaryote animals, constituted by orthophosphate residues linked by high-energy phosphoanhydride bonds, having distinguishing functional properties [41]. This polymer present in the skeleton of marine sponges acts as an extracellular system for storage and delivery of metabolically useful energy, also having an active functional role in extracellular reactions of the bone biomineralization, providing a source of energy.

A study developed by Wang and colleagues evidenced that both biosilica and polyP inorganic polymers have a positive effect on the differentiation of human multipotent stromal cells (hMSC) in the different osteogenic or chondrogenic cell lineages. In fact, the gene expression of bone morphogenetic protein 2 (BMP-2) and alkaline phosphatase (ALP) was significantly increased by these polymers, mainly in the osteogenic cells. Additionally, both proteins were considered morphogenetically active additives as these are capable of upregulate the levels of collagen type I and type II transcripts, in osteogenic and chondrogenic cells, respectively [9]. Also, it was reported that the mineralization of osteoblast-like SaOS-2 cells is enhanced when the cells are exposed to the inorganic polymer polyP [42].

As demonstrated, polyP can be used in varied applications in the field of regenerative therapies of bone diseases and bone repair, with different biomedical purposes. Its utilization may be a useful tool for the development of novel bone biomimetic strategies [39].

2.2.3.3 Collagen and Spongin

Collagen is a ubiquitous protein with multiple functions in invertebrates and vertebrates, proven to be a versatile material with many applications in several fields as food industries, cosmetics, pharmaceutics, drug delivery, and tissue engineering. Collagen is the most abundant protein of the body, present on the extracellular cell matrix (ECM) of tissues including ligaments, skin, tendon and bone in human. In bone, this fibril protein is highly abundant and representing 90% of bone organic mass with importance in mineralization herewith calcium phosphates [43].

The extraction of collagen from marine sponges have been studied by different authors, developing methodologies that differ from the ones commonly used for mammals or fish, due to the supramolecular organization of collagens in marine sponges. In fact, acidic treatments are scarcely effective for the isolation of collagen from marine sponges, while the use of neutral to basic solutions, in the presence of chaotropic agents as urea and/or salts is being much more efficient, namely from marine sponges species known to be rich in collagen, as *Chondrosia reniformis*. The presence of collagen-like protein (spongin) in the skeletons of demosponges has been also a remarkable object of study.

Collagenous materials have been isolated from marine sponges as *Axinella cannabina* and *Suberites carnosus* [8]. In sponges, the compaction of collagen fib-

rils and filaments produce the spongin protein collagen, which has high porosity, thermostability, and mechanically rigid structures, being very handy for tissue bionics [44]. The spongin is a short-chain molecule that shares features with basement membrane collagen type IV [45] and the spongin organization has been reported as analogous to the human collagen type XIII [46]. Spongin was ascribed being more resistant to enzyme degradation than collagen [47]. Recently was reported the use of spongin-based scaffolds isolated from marine Demosponge *Hippospongia communis* as a 3D template for the hydrothermal deposition of crystalline titanium dioxide and also as a novel carrier for laccase immobilization for the removal of various bisphenols from water solutions [44, 47].

2.2.3.4 Chitin

Chitin is the second most abundant biopolymer (right after cellulose), being present in cell wall of some fungi but mostly on skeletal structures of arthropods, and mainly exploited from crustaceans shells, namely from shrimp and crabs. This biopolymer is thermostable, natural, non-toxic, biocompatible, and biodegradable with good mechanical properties. Chitin can be easily tailored for specific applications as drug delivery, wound healing, gene therapy and tissue engineering, enabling the development of nanoparticles, nanofibers, membranes, sponges, gels and scaffolds [48].

Interestingly, chitin is an integral component of skeletal structures of plenty invertebrates, including marine sponges. Chitin was found as a component of skeleton in sponges of the order Verongida in 2007 [49]. Recently, chitin has been reported and identified in the skeletal structures of four families of sponges related to the order Verongida (Demospongiae) and the *Farrea occa* glass sponge [7, 50]. Currently, the identification of α-chitin from the *Suberea clavata* demosponge of the Aplysinidae family may be a useful taxonomic tool for the identification of unknown demosponges species that possess chitin as a component of their skeletons [50]. Also, poriferan chitin may be a versatile template for fabrication of extreme biomimetic materials where high temperatures and pressures enable the development of remarkable bioinorganic composites with applications in water filtration, biosensing and regenerative medicine [51]. The skeleton of *Aplysina aerophoba* was used as an extreme biomimetic 3D-α-chitin scaffold material effectively mineralized under hydrothermal conditions (150 °C), using ammonium zirconium (IV) carbonate as a precursor of zirconia [52]. An example study of the sponge based chitin-scaffold showed exceptional properties such as diverse structural architectures, physico-chemical properties, ion absorption, great hydration and interconnected channels with different purposes toward biomedical applications, as mucoadhesive nature or anticancer, osteogenic and growth factor deliveries [48].

2.3 Biomaterials

Novel compounds isolated from marine species are increasingly sought, being a promising and inestimable source for the production of biomaterials for biomedical application, namely in regenerative medicine strategies. The classical approach counts on a design to provide architectural framework to potentiate cell growth and adhesion to the constructed scaffold, possibly in combination with other bioactive molecules, as cell growth factors, to ultimately promote the tissue regeneration.

At least a thousand marine sponges species and eleven sponge genera such as *Haliclona*, *Petrosia, Cryptotethia* and *Discodemia* have been studied and identified as source of bioactive secondary metabolites. Among these bioactive compounds, one can find potent tumor-inhibiting arabinosyl nucleoside, anti-cancer, anti-malarial and anti-inflammatory effects, with relevant biomedical application [2]. One can thus imagine the combination of bioactive compounds with structural macromolecules addressed in the previous sections for the development of functional biomaterials. Besides, recent advancement in biomaterials development and biofabrication is pushing the exploration of novel strategies on design of materials with biomimetic properties, i.e., learning from Nature unique strategies to inspire astonishing designs and/or introduce exquisite biological effects into therapeutic approaches. In this view, biomaterials inspired in marine species have been tailored, exploring not only the biological structures of marine sponges but also their chemical composition, aiming tissue remodeling towards bone regeneration [2, 53, 54]. Hence, sponge origin collagen has been used to develop and fabricate new biomaterials, benefiting from its properties such as low toxicity, biocompatibility and biodegradability Moreover, the basal spicules of hexactinellid sponges reveal a set of physiological features and optical properties as the size, durability, flexibility and triboluminescence, suitable for the development of new marine-derived biomaterials [48, 55]. These and other examples will be discussed in the following sections.

2.3.1 Bioceramics

Bioceramics are a large class of fully, partially or non-crystalline ceramics conceived for repair and reconstruction of injured parts of the body, having tremendous potential as biomaterials mainly for scaffolds tailoring. Bioceramics categories include the calcium phosphates (Ca/P) as hydroxyapatite (HAp), the bioactive glasses and the glass-ceramics. Based on their tissue response bioceramics can be classified as: nearly inert (e.g., alumina), bioactive (e.g., bioactive glass) or resorbable ceramics (e.g. α-tricalcium phosphate) [56].

This class of materials is primarily used in low- or non-load-bearing and in compressive load applications due to their particular mechanical properties. Their mechanical rigidity coupled with their inorganic nature makes bioceramics fitting for

the repair and regeneration of hard tissues like bone and teeth, field in which these materials are employed for years [57].

However, inert bioceramics have some limitation caused by the formation of a fibrous capsule on the surface of the clinical implants, which prevents the bonding between the implant and the host tissue, thus hindering their use as suited scaffolds in the truly and fully repair of bone tissue, i.e., the integral regeneration of bone tissue. Contrarily, bioactive bioceramics are relevant to manufacture scaffolds for tissue engineering, revealing the capability of establishing bonds between the implant and the tissues, a critical step for the successful clinical treatments in regenerative medicine. In vitro and in vivo experiments revealed the formation of a layer of apatite in the glass surface responsible for bone bonding and repairing [57]: bioactive HAp or HAp-like coating layers are formed by a series of chemical reactions, supporting osteoblast cells adhesion, growth and mineralization, thus favouring bone tissue engineering strategies [58].

Furthermore, some bioceramics like HAp can be used for the development of permanent devices while others, such as some bioactive glasses, are degraded and reabsorbed by the body and their ionic dissolution products (e.g. soluble silica and calcium ions) can exert therapeutic effects like enhanced osteogenesis and induced angiogenesis [59–61]. In the latter case, scaffolds have the additional advantage of, if properly tuned, degrading at the same rate as the natural host tissue is replaced, leading to the total regeneration of the host tissue and the disappearance of the scaffold [6, 62]. In addition, bioactive glasses are also considered a promising application for stimulating the soft tissues' regeneration processes, including wound healing and angiogenesis mechanisms. The development of biomaterials for soft tissue engineering may benefit considerably from the bioresorbable features of some of these bioceramics [63].

Despite the recent advances in the field and the new technologies developed, mimicking the human bone porosity is still one of the main hurdles to overcome, due to the complexity of achieving suitable connectivity and tortuosity. Therefore, finding naturally occurring porous structures capable of being used as scaffolds or of providing templates for the production of innovative biomaterials is of utmost importance [64]. The employment of 3D structures from marine origin aiming for biomedical and biotechnological applications is not a new trend, as various animals have been used as 3D biomatrices in the last years such as sea urchins, coral skeletons and sponges [65, 66].

However, marine sponges remain overlooked regarding their bioceramics and the use of their body architecture as bioactive 3D bioceramics structures, when compared with other marine species [67–69]. Nevertheless, the use of marine sponges as precursors in the fabrication of ceramic-based scaffolds for bone tissue engineering has already been demonstrated [70, 71]. Using marine sponges as sacrificial templates for tissue engineering scaffold production is yielding very encouraging results, as the produced scaffolds preserve bioactivity, are non-cytotoxic, present adequate porosity and pore interconnectivity for cell proliferation and are able to achieve superior mechanical properties than the conventional bioactive glass scaffolds prepared with polyurethane [72]. Additionally, bioactive ceramics have the advantage of adsorbing

and potentiating the role of growth factors and cellular ligands, a vital step toward tissue repair [73].

In fact, Cunningham and colleagues were able to produce tissue engineered bone scaffolds from the calcinated marine sponge *Spongia agaricina*, after submerging the specimens in an 80 wt% HAp solid loaded slip and drying them. The obtained Hap-based scaffold displayed an overall porosity of 56–61% with 83% of the pores ranging from 100 to 500 μm (average pore size 349 μm) and an interconnectivity of 99.92% [70]. In another study, performed by Boccardi et al., *Spongia lamella* and *S. agaricina* were used as sacrificial template materials. The marine sponges were immersed in PVA-water solution concentrated up to 40 wt%, dried, and immersed in bioactive glass powder and finally calcinated. The developed marine sponge scaffolds presented higher mechanical properties than those made by foam replica method due to a decrease in porosity (68–76%), without affecting pore interconnectivity (>99%). Noteworthy, the produced pore structure possesses pores with a diameter in the range of 150–500 μm, required for bone ingrowth, and also pores ranging from 0 to 200 μm, necessary for neovascularization, which are very difficult to obtain through artificial techniques [72].

In summary, even with new advances in the processing methods that allow to better control the 3D architecture of the scaffolds, employing the calcinated skeleton of marine sponges provides an exceptional 3D porous and interconnected structure directly and without further processing, while presenting an inimitable bioactive surface. Despite the promising results obtained so far, marine sponge bioceramics are yet to reach their full potential. Nevertheless, research in the area is continuing as marine sponge bioceramics present a promising future for tissue engineering. More will be discussed further on, when addressing the use of marine sponges as natural scaffolds.

2.3.2 Composites

A composite usually is produced using two or more compounds, which combined lead to the enhancement in the chemical, mechanical and physical properties of the final material. Hence, a proper composite is, for instance, a combination of hard and soft materials that balance the properties of toughness, strength and water content, having significant effects on the mechanical properties such as a better fatigue resistance and resiliency [74].

Likewise, hexactinellid sponges have spicules structures constituted by a hard material of hydrated silica and collagenous soft material that have highly flexible and tough characteristics [75]. Recently, research into biocomposites has been focusing in this class of marine sponges, more specifically in *Euplectella aspergillum*, *Hyalonema sieboldi* and *Hyalonema populiferum* species. These glass sponges represent an excellent biomimetic model to develop new inorganic-organic composite biomaterials inspired in the morphological, optical and mechanical features of their spicules [76]. In 2007, a silica-chitin composite biomaterial was tailored for the first

time, mimicking the skeletons of the marine sponges [49]. Pallela et al. developed a tri-component scaffold system using marine origin biomaterials from the sponge *Ircinia fusca* (spongin/collagen) and the fish *Thunnus obesus* (chitosan and HAp) by freeze-drying (lyophilization) method. Comparison among chitosan, chitosan-HAp and chitosan-HAp-collagen scaffolds demonstrated that the tri-component scaffold had the highest thermal stability due to the presence of HAp and collagen, which are very stable. Furthermore, the developed scaffolds presented a higher in vitro cell proliferation using bone MG-63 cell line and an interconnected porosity (50–170 μm), proving to be an encouraging biomaterial for bone tissue engineering [77]. Arey and colleagues studied *Hyalonema* spicules and discovered that rigid natural composites, comprised primarily by a ceramic phase and containing an organic phase capable of fibril formation, would exhibit a viscoelastic behavior, contributing to mechanical energy dissipation, system function and survival to large deformations [78].

Therefore, it is clear that sponges represent an outstanding model for the biomimetic synthesis of 3D composites that present specific mechanical, optical and bioactive properties appropriate for the application as biomaterials.

2.3.3 Hydrogels

Hydrogels are three-dimensional polymeric networks made from hydrophilic, natural or synthetic polymers crosslinked by means of different mechanisms, rendering an insoluble polymeric matrix, capable to retain a large amount of water. This highly hydrated polymeric network constitutes an extensive framework for cellular proliferation, adhesion, and survival [79].

Indeed, hydrogels are biocompatible materials that mimic mainly the physical properties of soft tissue. These materials have many applications in regenerative medicine, namely tissue engineering, as well as on drug delivery [80], due to its flexible synthesis using different methods and an extensive range of components. They are based on synthetic polymers as poly(ethylene glycol) (PEG) [81] and poly(vinyl alcohol) (PVA) [82] or biopolymers and biomacromolecules such as collagen, agarose, alginate and chitosan (derivative of chitin) [83–86], among many others. Synthetic materials often have high toxicity in vivo and poor biodegradability and for these reasons, the biological polymers are more desirable over synthetic ones.

Collagen is itself a biomimetic material that has been used to the development of a vast number of biomaterials, as collagen-based hydrogels for tissue engineering. Although the main sources of collagen are still bovine and porcine skin and tendons, some ethical and religious concerns have been arising, namely the association to disease transmissions and potential immunogenic reactions [87]. Since the use of collagens for tissue engineering applications should be free of these type of issues, alternatives are being pursued and collagens isolated from marine organisms may be an alternative and sustainable option to be addressed [88], including from marine sponges as abovementioned. *Chondrosia reniformis*, a species of marine demosponge, consists predominantly of collagenous tissue that presents labile inter-

fibrillar crosslinks, and this feature can be employed in many pharmaceutical and biomedical applications as injectable collagenous hydrogels [89].

Interestingly, different extraction methodologies to obtain the collagenous material have been reported in *C. reniformis*: the collagen/gelatin method using green solvents with water acidified with pressurized carbon dioxide [90, 91] and the novel method that extracts collagen/Glycosaminoglycans (GAG)-like molecules and other proteins, using a pre-treatment step with phosphate buffer saline/ethylenediaminetetraacetic (PBS/EDTA) and an incubation step with disaggregating solution, that yields a high collagen content with new rheological properties [89]. Nevertheless, the standard methodologies to produce collagen from this sponge species are based in chaotropic agents, as the ones developed by Swatschek et al. [92] or based on the method developed by Matsumura et al. for echinoderms [93]. This collagen can be used on the development of microparticles for drug delivery [94] or membranes for biomedical application [95]. Another study approaching cartilage tissue engineering suggested the incorporation on hydrogel in sponge architecture structure as strategy to increase structural stability and improve performance as scaffold [11].

2.3.4 Porous Marine Sponge as Natural Scaffold

The nanostructural organization of the body of the marine organism is an endless source of inspiration for offering multiple technical solutions in aerodynamics, bionics, architecture, and fabrication of biomaterials [96]. The body structure of marine sponges could be used as natural porous scaffolds that act as a support for the development of studies in replacement of bone and tissue engineering approaches. The skeletons of sponges are well-organized and functionalized structures with three-dimensional (3D) hierarchical architecture, adequate for the seed of human stem cells. These skeletons provide a support for the cells, but also an information to be used in the fabrication of synthetic tissue-engineering scaffolds, giving the biochemical cues provided by their composition [46].

Numerous studies have been undertaken to investigate the potential of marine sponges as natural scaffolds. According to Green et al. [46], the *Spongia* skeleton was used to be an affordable scaffold to test the hydration potential of spongin fiber network as an open porous fiber framework, which enables the human osteoprogenitor cells attachment, aggregation, and proliferation. Subsequently, histological examination showed the formation of the bone matrix. Results of the previous study confirmed the potential of sponge skeletons as a delivery scaffold for osteogenic factors and a sustainable source for bone tissue grafts and tissue regeneration [46].

Similarly, the potential of *Biemna fortis* marine sponges was studied as bioscaffold in bone tissue engineering and bone augmentation. The bare sponge scaffolds were produced by heating the specimen in a furnace at 1190 °C and further incorporation of two growth factors (IGF-1 and BMP-2). Results demonstrated that the skeleton of *B. fortis* showed a fibrous network with a 10–220 μm internetworked

porosity [53]. Recently, a study with *Aplysina aerophoba* sponges as a 3D chitin-based scaffold demonstrated their potential use in tissue engineering. In in vitro assay study, the 3D biomaterials were cytocompatible and human mesenchymal stromal cells (hMSCs) attachment, growth and proliferation were confirmed by the increase of metabolic activity, cells number and differentiation into chondrogenic, adipogenic and osteogenic lineages [11]. This research revealed the potential of marine sponges as a scaffold and a novel source of chitin to be used in modern therapeutic tools for tissue engineering and biomedical purposes.

2.4 Biomedical Application

The marine environment is an exceptional source of new materials widely used for several biomedical applications due to their resemblance to the native extracellular matrix of human tissues and known safety origin to isolate compounds. The marine sponges in particular may be used as unconditional bioinspiration, as it is one of the most striking Nature models. As previously stated, the skeletons of marine sponges have high porosity and interconnectivity due to the presence of the canal systems, which provide a favorable environment for seeding osteogenic cell lines or hMSCs [11, 46]. These remarkable morphological structures can thus enable biomimetic approaches, especially for the fabrication of bone tissue grafts and regenerative therapies tackling bone diseases [53, 54].

Another inspiring feature of some marine sponges is the biosilicification process by which amorphous silica is generated from silicic acid esters by the silicatein enzyme, generating silica skeleton and spicules [97]. The controlled deposition of silica is of great potential importance in tissue engineering, as it would allow the modulation of the speed and amount of tissue regenerated during bone regeneration and tooth reconstruction in vivo and at mild physiological conditions. In order to possibly create via the biomimetic approach tailored biosilica structures the enzymes associated with this process, silicatein and silintaphin-1 and -2 (silicatein specific interactors), would have to be tightly controlled and balanced [98]. Wiens and collaborators, using recombinant silicatein and silintaphin-1 at equimolar concentrations, were able to produce biomimetic filamentous protein structures resembling natural axial filaments, establishing the basis to control in vitro biosilicification [99]. Furthermore, if the direct assembly of silica was controlled by the combined action of these enzymes and since spicules have the ability to transmit light efficiently [35], it would be possible to create micro-structured light-guiding composites that could be an economical substitute of industrial glass fibers. These composites can find applications in electronics and biosensors practical to the biomedical field. In an interesting work developed by Natalio and colleagues, an 8-Glu tag was added to the N-terminus of silicatein, conferring this protein HAp-binding capacity. The immobilized tagged proteins promoted the directed formation of biosilica coatings on synthetic HAp nanofibrils and dental HAp, after the addition of biosilica precursor [100]. This “smart glue” has the potential to seal surface defects, which would allow

a reduction in the possibility of bone and tooth decay and of dental hypersensitivity, as well as to coat metallic implants with the intention of enhancing their biocompatibility or to encapsulate and release in controlled manner drugs or other bioactive compounds.

The presence of calcium carbonate ($CaCO_3$) in the spicules of some marine sponges is a noteworthy characteristic. Calcium carbonates are synthesized during the early hydroxyapatite-based bone formation and represent a component mineral in the matrix of the vertebrate bones. Also, the calcareous sponges own calcium carbonates skeletons where the inorganic elements, as spicules, are formed by means of an enzymatic reaction of carbonic anhydrase(s). Otherwise, in human, the bone mineralization mechanism is a highly regulated process and in case of flaw could promote the calcification in soft tissues (for example kidney stone formation), osteoporosis or dental dysplasia diseases, just to mention a few. The quinolinic acid sponge metabolite was capable of activating the carbonic anhydrase and enhance the mineralization of human bone cells [42], demonstrating that marine sponges can synthesize diverse metabolites with high applicability in human bone mineralization.

Nowadays, the demand for use of natural biodegradable materials has been growing in the scientific community and companies in the pharmaceutical field to take into consideration the requirement of highly innovative materials and technologies. The marine environment could be a viable and sustainable source of innovative biomaterials as the valorization of the marine species, like marine sponges, can be an alternative resource supply. Regarding the valorization of these resources, the natural skeleton of four marine sponge species was used as bio-based dressing in form of a powder or polymeric film shapes for drug delivery (L-cysteine hydrochloride) applications. Results pointed that the content of glycosaminoglycans (GAG) anchored in the skeleton of the natural sponge could act as bioactive-biomimetic carriers, regulating the wound healing mechanisms. This newly developed marine sponge natural biomaterial may be a sustainable alternative to the polymeric spongy-like matrices developed and available on the pharmaceutics market [101].

Demosponges, in association with microbes, are the major producers of pharmacologically important bioactive compounds. The novel leading compounds with clinical and pharmacological importance have been isolated from the symbionts actinobacteria living with marine species, and sponges-associated actinobacteria are the largest source of natural products with unusual biological activity [102]. Also, in a situation of nutrient competition and of a limited living space, the *Micromonospora* sp. marine bacteria associated with sponges could fabricate manzamines, a group of sponge-derived alkaloids. These compounds possess a high potentiality in the pharmacological field, with application in tuberculosis, HIV and malaria diseases [103]. Thus, the potential is huge and the story of marine sponges in the biomedical arena has just start to be written.

2.5 Future Remarks

The Ocean and marine resources are a source of widely unique natural products that display a wealth of biotechnological application, presenting a high potential for human health applications in the pharmaceutical and cosmetic industries, as well as in regenerative medicine, particularly when considering tissue-engineering approaches relying on the use of biomaterials. Indeed, the value of marine sponges as resources for new macromolecules and bioactive compounds is magnificent. The investigation of the micro-architectural and cell organization in sponges is central to tailor-made a biomimetic approach inspired by these important models and a sustainable alternative to the synthetic materials. The development of biomaterials inspired in natural models has a huge biomimetic potential, not yet completely explored. Therefore, it is required the improvement of our knowledge underlying the ecology, cell organization, reproductive biology, life spans and structural features of marine sponges. The research in marine 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 calcium phosphate or polymer scaffolds, as templates for producing biomimetic ceramic scaffolds or directly as osteoconductive grafts. However, these organisms present a significant drawback that is the sustainability aspect, due to ecological limitations (rare species and part of ecosystems that should be preserved, which is typically incompatible with harvesting) or technological constraints (sponges species are in depths that increase significantly the cost and requirements for their collection). A possible solution is the development of marine sponges farming techniques or the transplantation of sponges back into areas where they become extinct. Hence, sponges are promising species in which researchers continually attempt to improve or develop novel biomaterials.

To conclude, these biomaterials offer excellent and unique qualities like biocompatibility, degradability and promotion of cell adhesion, differentiation and proliferation. Advances in biomimetic materials research for tissue engineering should be inspired in Nature's materials as well as in marine structures since they were tested and developed for millions of years of evolution and present brilliant solutions and smart designs for our most complex challenges. Despite being ancient organisms, there is yet many blank pages on the book of marine sponges, but scientists across the world are cooperating actively to write some words and sentences. Ultimately, a story will come and their biomedical use will be a clinical reality.

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Chapter 3
Marine Derived Biomaterials for Bone Regeneration and Tissue Engineering: Learning from Nature

Besim Ben-Nissan, Andy H. Choi and David W. Green

Abstract Marine structures, biogenic materials, and biomimetic approaches applied to the fabrication of advanced biomaterials and implants are used to address the shortcomings of existing scaffold designs that are biologically un-responsive throughout the regeneration process and lack necessary versatility. Bioactive ceramics converted from biostructures or natural marine-based materials such as corals, sea urchin, sponges and shells are being designed into functional scaffolds that can adapt and evolve to changing environment during regeneration process. They can regulate cell responses at nanostructured surfaces, and as modules for self-assembling by the patient's own cells and as smart devices that possess tissue specific homing capabilities. These natural structures can be converted to bioactive ceramics such as hydroxyapatite to assist osseointegration. This chapter covers biomimicry, evolution of marine structures, and their specific use and current research on natural materials such as coral, sponge, sea urchin, sponge nacre, and foraminifera as models and raw materials for bioactive bone scaffolding materials and tissue engineering.

Keywords Marine structures · Hydroxyapatite · Bioactive · Biogenic · Biomimetics · Coral · Nacre · Bone grafts · Scaffolding

3.1 Introduction

At present, synthetic implants suffers a major drawback of being incapable of adapting to the local tissue environment [1]. Therefore, it has been accepted that new advanced bioactive materials are needed that can elicit regenerative responses.

Nature can inform and direct us in many incisive ways on how to build structures, design architectures and fabricate materials with exemplary high performance using

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minimal energy and substances which are optimized for many different functions. During the last few decades, studies on natural engineering structures or biomimicry has provided various means of generating unique scaffolds that can be applied in regenerative medicine with the potential to outperform conventional advanced man-made functional materials. According to Vincent et al., biomimicry research essentially involves abstracting good design from nature [2].

One of the main approaches towards driving tissue regeneration is the use of biochemical factors to trigger cell proliferation and differentiation. Bone morphogenic proteins (BMPs) are widely utilized in musculoskeletal tissue engineering to promote bone tissue formation and gene expression [1]. Gaining a deeper insight into the functions of growth factors and the role they play during tissue regeneration has increased development of pharmaceutical grade growth factors for use in clinical applications. Other biological factors with unique cellular functions and activities are sought to establish more precise control of the regenerative response and simulate the sequential temporal and spatial secretion of secretory factors (proliferative, differentiation and growth). There is a clear and present need for better tissue engineering scaffolds that possess more natural bio-responsive environments conducive to guiding the natural processes of regeneration which can be highly intricate and dynamic in space and time.

We contend that there needs to be a gradual change in the scaffold environments that are more bioactive and responsive, whereby the synthesized biomatrix evolves in real-time to meet the demands and optimized to the adaptive growth and regeneration of human tissues. Despite the well-known fact that cells proliferate and differentiate, they do alter their environment during the process. Consequently, any advanced biomimetic scaffolds produced in the future must be able to adapt to these changes and meet the ever-changing needs of developing tissues. It has been anticipated that we can synthesize biomaterial scaffolds with functional cross-links and pendant side groups that interact with surrounding integrated cell populations at various levels that includes the surfaces, at the architectural or morphological levels or at a functional level. Marine structures fill this gap due to their chemistry, surface arrangement and their unique architecture.

Development of modular self-assembling biomaterials is an attempt to harness the cell as the master-builder of its own extracellular matrix using tailored synthetic components with high energy efficiency is relatively new [3].

The way natural systems allocate energy for different functions can instruct us on how to best optimize the design of materials and structure with minimum energy and with control at the molecular level.

In essence, natural material can out-perform their synthetic counterparts simply by paying more attention to the design and the properties of self-assembly. Furthermore, the utilization of a new generation of composites can also provide an enhancement in performance through the regulation of their mechanical properties.

A “bottom-up” chemical process is used by natural organisms as a method to produce materials over many dimensional scales. By harnessing these processes and particularly those in the nanoscale, we have the potential to create “living materials” that adapt and respond to their surroundings. There is now a greater appreciation

and understanding to the significance of bio-inspired and nanotechnology-based approaches to the fabrication of new bio-related materials. The materials component of tissue engineering is the most amenable to this type of analysis alongside the increased sophistication of materials chemistry and nanofabrication to provide exceptional biomimetic solutions.

In this chapter, we will outline the development and progress of marine structure based on biomimetic and nanoscale approaches in regenerative medicine and hard tissue engineering providing successful worked examples from our own research and fellow researchers with particular emphasis on inorganic structures.

3.1.1 Biomimetics

Tissue engineering is a multidisciplinary area with the intention of recreating fully functional and healthy human tissue and organs customized for patients who is suffering from a loss of hard or soft tissues. The greatest amount of progress has been made in growing and nurturing tissues in artificial culture and harnessing candidate biomaterials that promotes and guide tissue formation in extended space and time. The most important approach towards driving tissue regeneration is the introduction of biochemical factors that triggers cell proliferation and differentiation. One such example is bone morphogenetic proteins (BMPs) and despite the fact that it is very expensive, it has been extensively used in musculoskeletal tissue engineering to promote bone tissue formation and gene expression [1].

Gaining a deeper understanding into the function of growth factors and the role they play during tissue regeneration has stimulated the development of pharmaceutical grade growth factors for clinical applications. Other biological factors with unique cellular functions and activities are sought to establish a more precise control of the regenerative response.

Less progress has been made on the development of scaffolds with desired properties, especially biological ones, which provide the physical shape and form for archetypal tissue formation. During the last two decades, the almost non-existent biomaterial scaffolds with broad applicability and bio-responsiveness specifically osteoinductivity is being addressed somehow with limited success by many research teams worldwide [4–6].

There is an increased emphasis on the use of biocomposites and native biopolymers such as collagen as well as fabrication protocols operating at the molecular and nanoscale levels which enables more precise control of physical and chemical properties in the final macrostructure [7, 8]. Such structures are designed to provide a better match to the naturally occurring ones. For example, electrospun materials offer fibrous constructs with dimensions analogous to native extracellular matrices and thus provide equivalent physical and chemical properties [9]. In order to control cellular activities, the physical and chemical surroundings play a pivotal role in the quality of tissue organization, formation and quantity, hence the focus on materials design and synthesis.

At the moment, we are learning from nature in terms of design, shape and materials [10]. Investigations into the way naturally occurring marine-based materials are constructed (from the organic and inorganic chemistry perspective) and the way they adapt to their environment will enable us to produce an exciting array of self-responsive structures and materials for regenerative medicine, structural applications and applied materials [11].

As stated earlier, in nature, biomaterials (specifically marine-based structures) are made with immaculate resource and energy efficient methods using common and readily available substrates through self-assembly into highly organized hierarchies. Biomechanically functional structures optimized to their environment are also manufactured using this approach. This gives us the opportunity to produce structures with intricate shapes and architectures that are tailored to their functions and do not break down.

Biomimetic approaches can yield promising outcomes for application in the tissue engineering of skeletal tissues. There are four general approaches:

1. The first approach involves configuring the material environment at the molecular and macromolecular levels in an effort to mimic native extracellular matrix. The hope is to further expand this ongoing research towards the design of clinically relevant scaffolds for regenerative medicine using a unique set of self-organizing hierarchical structures designed and synthesized according to biological principles of design. In the meantime, we are finally taking the initial steps towards this goal. We have begun harnessing materials from marine structures with unique designs and shapes (as nature does with enormous precision and control over inorganic molecules and nanoparticles) to construct advanced functional structures. These structures and materials can be adapted to clinical environment with intended applications in tissue engineering and bone regeneration as well as in pharmaceutics such as locally targeted slow drug and gene delivery.
2. The second approach is more sophisticated in that it seeks to develop new chemistry that simulates biomineralization to generate unique structures with the exquisite complexity of their natural counterparts [12, 13]. While the underlying mechanisms of biomineralization are not well understood, some advances have been made using analogous chemistry, biomolecules (collagens, amino acids) and bio-templates formulated with calcium phosphate and silica substrates [14–16].
3. The third approach uses nanoscale manipulation and processes to simulate, in part, the nanoscale control of materials formation in biology [17]. Now, some progress has been made in duplicating a small range of biologically analogous structures in the laboratory, where they displayed limited hierarchical organization. Calcium carbonate and calcium phosphate structures produced using these unique chemical systems provide ideal scaffolds for biomedical implantation.
4. A fourth strategy involves controlled mineralization surrounding the modified organic matrices derived from natural skeletons [18]. These techniques replicate, in part without genetic control and biopolymer/sugar moiety inclusions, the biomineral pattern formation of carbonates that occurs so readily in nature. Microemulsions provide equally soft, sculpted morphologies for min-

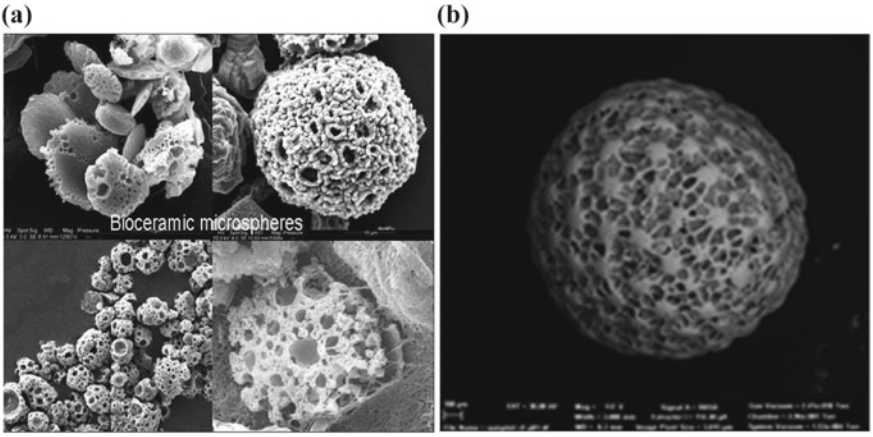

Fig. 3.1 Synthetic porous microspheres with modulated morphology, architectures and sizes generated using techniques in biomimetic materials chemistry (**a**); in comparison to foraminifera a natural microsphere shell structure (**b**)

eralization into complex forms. Surfactant foams or polymer beads are utilized to create porous hollow shells whereas biocontinuous microemulsions produce microskeletal frameworks [19]. Marine shells are molded around transient dynamic arrays of vesicles and lipid sacs. These components provide concentrated sources of biomineral precursors.

3.2 Scaffold Designs

During the last three decades, the above-mentioned approaches have been taken into consideration during the design of biomimetic scaffolds. The most promising approach has been: "to allow nature to do the work" by using and adapting structures sourced from marine environments, such as invertebrate skeletons (both organic and inorganic). In this way, complex forms and architectures can be harnessed for biomedical applications [20] (Fig. 3.1).

The marine environment is uniquely rich in skeletons with architectures and chemical composition suitable for human implantation. Coral skeletons and converted coralline calcium phosphates are exquisite examples. They have demonstrated substantial clinical success as a template for tissue reconstruction [17, 21, 22]. This has motivated researchers to look for other skeletons with better mechanical properties such as hydrozoans as unique potential candidates for tissue engineering of mineralized tissues [23].

The unique 3-dimensional structure supported growth and enhanced differentiation of stem cell progenitors into bone cells unlike standard carbonate frameworks which do not induce stem cell differentiation [24]. It was suggested that the 3D

topology and specific surface features of hydrozoans instigated faster cell adhesion, proliferation and differentiation. More research is needs to be carried out to determine the exact mechanism of action between the natural material and cell.

3.2.1 Natural Evolution

In the medical field, the translation of products from nature into technology is one of the most basic and powerful means of resolving technological and scientific problems and conflicts with proven success. Natural history collections are a unique and rich source of practical ideas and solutions for the initial stages of tissue re-assembly in artificial culture. This may seem counter-intuitive as it is wholly independent of human structure, but it potentially provides more simplified instructions for newer designs and synthesis.

Understanding the evolution of tissues and organs as well as their design and function will provide an undiscovered route for creating elements that can be used to reconstruct tissues in the simplest and most practical way possible. This approach is often termed evolutionary tissue re-assembly or Darwinian tissue engineering. Fossilized organisms have also been used as relevant models for providing new materials. While nature cannot produce the perfect designs, it can generate the most ideal, optimized and functional adaptive ones.

Biological structures and biomaterials have evolved by natural selection over many millions of years of strict conditioning, smoothing-out trade-offs between conflicting demands and limitations of an environment in order to maximize fitness and function. These conflicts are omnipresent but can be partially resolved to generate extremely well-functioning materials. The end results are biomaterials with compromises that exhibit high levels of performance made with minimum use of energy.

A Darwinian approach to regenerative medicine may also provide a wealth of information on the design of new replacement hard and soft tissue for both orthopedics and maxillofacial surgeries. The evolution of tissues by natural selection provides us with a view of how different strategies of development have been harnessed by organisms according to function. As a result, we should be able to provide simplified assembly strategies to recreate functional approximations of every human tissue.

At present, living cells has not been fully utilized or applied in the manufacture of bio-inspired materials where they can provide specific genetic instructions to guide the construction of biological structures and materials with tailored functions. Consequently, one of the most fascinating bio-inspired approaches is to use cells and organisms directly to literally grow biomaterials according to our requirements and specifications in a test tube or beaker [25]. This can be achieved by judicious modulation of the growing environment.

Single-celled organisms such as Diatoms, Foraminifera and Coccolithophores are a convenient starting point as they are the most rudimentary and elementary organisms to grow and support in artificial culture and provide enough flexibility for proving this approach as practically beneficial. Diatoms are of great interest for the

development of new strategies in nanotechnology and molecular assembly as they provide modes of construction at these scales that could benefit the development of lab-on-chip biosensors. Diatoms have even been described as "natural born lithographers" in recognition of the technique [6]. A number of researchers are exploiting the mechanisms of patterning by diatoms to use in patterning microchips. Others have suggested using them as drug eluting modules because of their beneficial microscopic size and reticulated internal pore structure. In the field of biomimetic photonic materials there is a vision to grow butterfly scales encompassing photonic crystals by exploiting techniques in genetic engineering [25].

Growing materials with living cells integrated during synthesis and construction is an attractive proposition in the medical field. Using this approach, the directed evolution may be possible with specific organisms that rapidly reproduce so that many thousands of generations are produced in short experimental periods. Protocols are well established now for the mass production of new proteins using a combination of site random mutagenesis followed by high-throughput screening [26].

The spatial and temporal orchestration of cells to form tissues and organs is genetically controlled during embryology [27]. Novel techniques in tissue engineering can be introduced using the principles of developmental biology. One such example, as proposed by Marga et al., is a scaffold-free approach that relies on directing cells and tissues to self-organize and self-assemble.

Synthetic tissue biology is a newly emerging discipline which seeks to engineer tissues and shape them into complex biological assemblages [28]. The reverse engineering of biological materials, systems, tissues, or organs is an example, which allows us to "decipher" the information regarding the ways these systems are constructed and how they operate at highest level of detail. Some has suggested that it will revolutionize the concepts and approach for re-engineering biological systems. Synthetic biology for forming multi-cellular tissues uses the most advanced methods available for building extracellular environments to direct morphogenesis of cells and tissues. Using a different strategy, cells are designed and constructed with novel functions and coaxed into multi-cellular organizations. There is also an aim to extend and modify the behavior of organisms (primarily unicellular ones because they are open to manipulation) and make them assemble and perform new programmed tasks, some natural and others to un-natural functions [29].

Seemingly, one of the most vital ideas about how nature constructs itself into the richness of shapes, sizes and forms is called tensegrity [30]. In the vast diversity of nature, there are countless identifiable blueprints concerning the construction and organization of cells and tissues into organs.

Evolution by natural selection has consistently created assembly rules and design solutions that have been conserved and re-applied in organisms throughout the gradual ascendency and emergence of new forms of life.

3.3 Marine Skeletons

Transformed coral (converted to calcium phosphates by hydrothermal treatment) has been the primary source of natural skeletons for bone tissue engineering due to its chemical, crystallographic and structural complementarity to native human bone since the 1970s [17, 31, 32]. Since then, researchers have made use of invertebrate marine skeletons of hydrozoans, cuttlefish [33], marine sponges [34], nacre seashell, echinoderm spines [35] based on similar methods.

From that point on, marine skeletons with its optimal ranges of pore sizes, channels, and structural networks have been used as templates for organizing and nourishing the growth of human tissues prior to transplantation. Furthermore, entire natural skeletons (without conversion to HAp) have also been used as templates for carrying biomolecules. Accordingly, diatom skeletons have been tethered with active biomolecules such as an antibody to be used in immunodiagnostics [36].

Mollusc shells are a fascinating model for understanding the complexities of biomineralization such as the control and regulation of protein-mineral interactions [37].

Considerable research efforts have been focused on the development of efficient and cost effective methods to produce different calcium phosphate phases from biogenic natural materials. It has been previously reported that a number of natural materials such as those of sea coral, seashells, sea urchins, nacre, Mediterranean mussel, land snails, and cuttlefish can potentially be utilized to produce calcium phosphate materials for biomedical applications because of their unique architectures as well as their composition, which is primarily calcium carbonate [38, 39]. These calcium phosphates include hydroxyapatite (HAp), tri-calcium phosphate (α- and β-TCP), tetracalcium phosphate (TTCP) and octacalcium phosphate (OCP).

Similarly, the skeletal ossicles from sea stars (*Pisaster giganteus*) have also been investigated as they provide an ideal architecture along with physical and chemical properties conducive to bone restoration [35].

During the past four decades, researches previously carried out has revealed that we have so far identified candidate biomatrices in nature, with varied chemical homologies and structural analogies to human extracellular matrices and whole tissues. Certain marine animal species has been selected and applied in the regeneration of human bone and cartilage. However, their full use in the regeneration of the abovementioned tissue as well as the regeneration of other tissues has yet to be harnessed and fully exploited.

3.3.1 Sea Urchin: Echinoderm Skeletal Elements

Sea urchin skeletal plates are punctured by a very regular series of pores. In *Centrostephanus nitidus*, approximately three quarters of the pores are exits for their spine feet. The pore diameter is approximately 200 μm at the spine bases and up to

600 μm for the tube feet, while the remainders are channels connected to the reproductive and alimentary systems and are very much larger to accommodate larger throughputs of fluid (1000–2000 μm in pore diameter).

Echinoderm skeletons are constructed from a unique, intricately shaped, 3D single crystalline meshwork with a topological structure in which every internal pore and channel is in direct contact with all others (periodic minimal surface). This property is likely to facilitate mass transfer and tissue development [40].

Studies has been carried out using the replamineform techniques for replicating perforate echinoderm structural elements. This approach shows promise in the generation of hard tissues for bone replacements as well as a potential candidate in the development of prostheses for trachea and blood vessels [41].

Conversion of these calcium carbonate structures to calcium phosphates such as HAp via hydrothermal processing transforms the echinoderm structures with mechanical and chemical properties equivalent to those of human bones [42].

The sea urchin spicule is a composite of organic and inorganic materials that the marine organism synthesizes using the most readily available elements such as Na, Ca, Sr and Mg found in sea water. The fully formed spicule is composed of a single crystal with an unusual morphology in 3D. It has no facets and forms a star-like shape (Fig. 3.2). It has been suggested that for sea urchin and other marine organisms to achieve such unusual morphologies, they must first deposit a disordered amorphous mineral phase and then let it slowly transform into a crystalline structure with neatly aligned into a lattice with a specific and regular orientation, while maintaining their morphology.

During their unique transformation from disordered amorphous structure to ordered crystalline structure, the sea urchin spicule is formed inside a clump of specialized cells and begins as the organism lays down a single crystal of calcite, from which the rest of the spicule is formed. It was observed that starting from the crystalline center, three arms extend at 120° from each other. The three radii are initially 40–100 nm size amorphous calcium carbonate but slowly converts to calcite.

The mechanism behind the conversion within this environment has not been fully comprehended but it was hypothesized that it might be due to a dissolution and ordered precipitation mechanism at known crystallographic orientations of calcite or aragonite [42].

Similar transformation can be observed in a sol-gel developed hydroxyapatite where the amorphous calcium phosphate is transformed to crystalline hydroxyapatite nanoplatelet via a thermally activated process [43].

Despite the fact that the transformation of sea urchin is carried out at ambient temperatures, this analogue transition in synthetic hydroxyapatite displays a plate-like morphological adaptation, where it is possible that no major changes in the structural shape can occur. With respect to the final product, transformation to crystalline phase from amorphous phase can take place easily and the shape of the platelets remains unchanged [44].

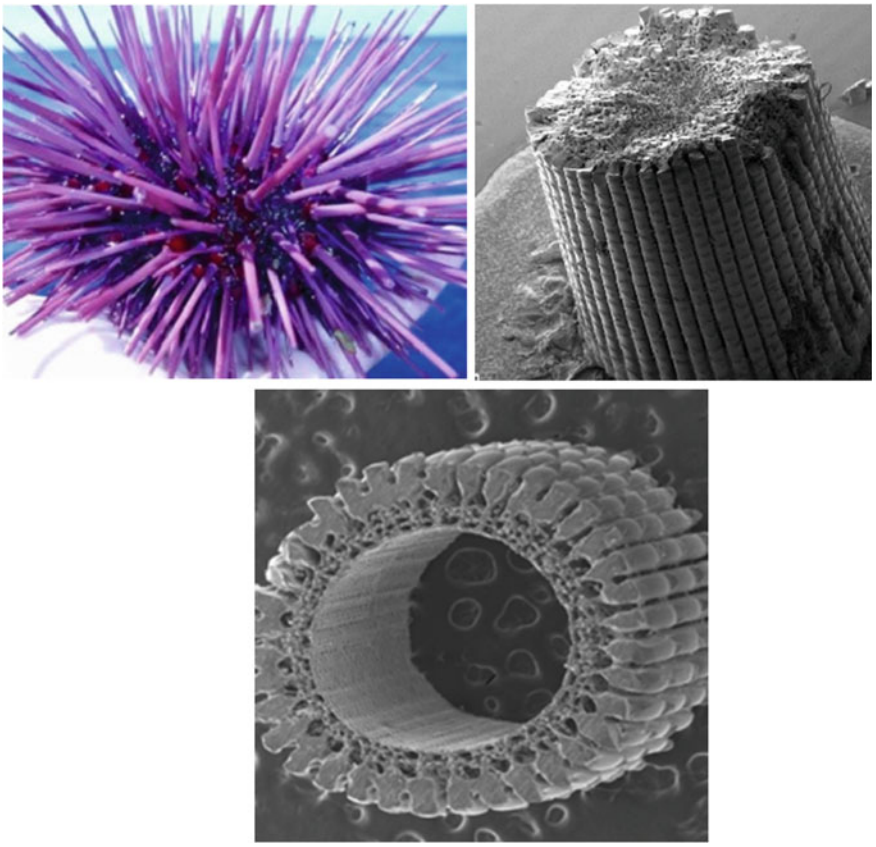

Fig. 3.2 Sea urchin (echinoderm) and scanning electron microscopy (SEM) images of their spicule structure

3.4 Coral Skeletons

Corals offer great opportunities in bone tissue engineering, either in their natural form or as hybridized synthetic forms [21]. Natural coral exoskeletons have been proposed and widely applied in orthopedics and dental and maxillofacial surgery as bone replacements due to their combination of good mechanical properties, open and interconnected porosity, and ability to form chemical bonds with bone and soft tissues in vivo.

In comparison to most porous calcium-based ceramics, corals and coralline structures have been discovered to possess similar or superior mechanical properties. In addition, their rates of resorption have been observed to be the same as the formation of new host bone tissues. According to Dauphin, the organic composition plays a vital role in determining the mechanical properties and biocompatibility of the coral. The

abundance and composition of the organic matrices are responsible for successful biological integration of natural unconverted coral with human host bone [45].

In tissue engineering and typical routine orthopedic surgery, the use of coral skeletons in conjunction with external fixation devices has so far been limited as they are inappropriate for load-bearing applications. This is the consequence of the higher dissolution rates of calcium carbonate structures compared to calcium phosphate ceramics.

Sol-gel coating technologies were used to enhance the strength of corals. This in turn increases the number of skeletal locations where they can be used including load bearing applications and slow drug delivery [46–51].

The osteogenesis potential of coral skeleton is greatly enhanced when they are combined with in vitro expanded stem cells (HBMSc) compared to pure scaffold or fresh marrow added to scaffold [24].

Results of in vivo segmental defect studies using large animals has revealed that there is a complete re-corticalization and formation of a medullary canal with mature lamellar cortical bone giving rise to clinical union in a high number of cases [21, 52].

The outcomes of structural and biomineralization studies of coral can be used to assist the development of new advanced functional materials because of the unique nanoscale organization of organic tissue and mineral as highlighted by Ehrlich et al. [53]. For example, the deep-sea bamboo coral exhibits bone-like mechanical and biochemical properties at a macro-structural level.

Acidic fibrillar, a specialized collagen matrix, shows potential as a model for future tissue engineering applications. The growth of osteoblasts and osteoclasts were supported by the matrix. Moreover, the collagen matrix (gorgonin) of this coral displays exceptional bio-elastomeric properties and could make them an ideal candidate as blood vessel implants [53].

Coral skeletons and converted coralline apatites have demonstrated substantial clinical success as a template for tissue reconstruction [21]. This has spurred on researchers to investigate other skeletons with better mechanical properties such as hydrozoans as a unique and possible candidate for the tissue engineering of mineralized tissues [23]. Their unique three-dimensional natural porous structure with nano, meso and macro pores enables bone growth and vascularization (Fig. 3.3). They also enhance the differentiation of stem cell progenitors into bone cells unlike standard carbonate frameworks that do not induce stem cell differentiation.

It was suggested that the 3D topology and specific surface features of hydrozoans promotes faster cell adhesion, proliferation and differentiation [52]. However, the exact mechanism of the interaction between hydrozoans and human cells remains unknown and gaining an insight into the action is required.

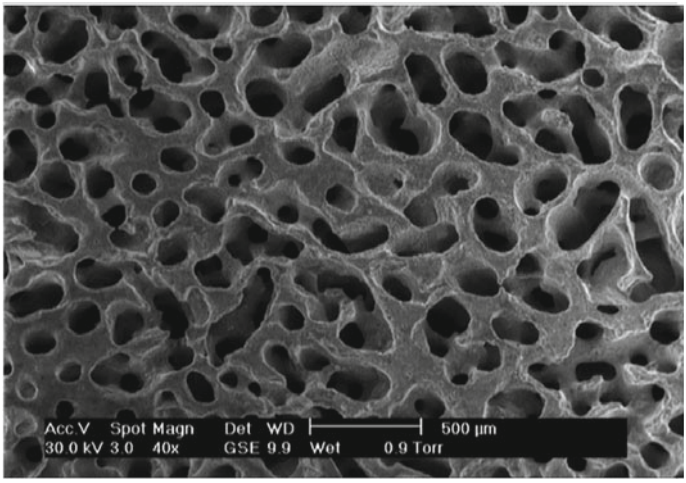

Fig. 3.3 Natural coral structure showing the different interconnected pores and its architecture

3.4.1 Properties and Applications

Natural coral graft substitutes are derived from the exoskeleton of marine madreporic corals. Attempts were first made in 1929 by researchers to use coral for dental applications. Since then, corals were recognized as a possible candidate as bone graft substitutes and was examined in animals in the early 1970s and subsequently in humans in 1979. The structure of the commonly used coral, Porites, is similar to that of cancellous bone and its initial mechanical properties resemble those of bone. The exoskeleton of these high content calcium carbonate scaffolds has since been shown to be biocompatible, osteoconductive, and biodegradable at variable rates depending on the exoskeleton porosity, the implantation site and the species. Although not osteoinductive or osteogenic, coral grafts act as an adequate carrier for growth factors and allow cell attachment, growth, spreading and differentiation. Natural coral exoskeletons have been observed to be an excellent bone graft substitutes if they are properly applied and selected to match exactly the rate of resorption with bone formation at the implantation site.

It has been widely accepted that the beginning of the coral life cycle begins with the polyps which absorbs the calcium ions and carbonic acid found in seawater and uses them to generate calcium carbonate in the form of aragonite crystals, which represents 97 to 99% of the coral exoskeleton. The remaining composition is composed of various elements and is dependent on the environment but mainly consists of trace elements of magnesium (0.05–0.2%), strontium, fluorine [54] and phosphorous in the phosphate form (0.02–0.03%) [55, 56]. These elements that are composed in the coral exoskeleton structure are known to play a critical role in the bone mineralization process and in the activation of key enzymes associated with bone cell remodeling.

Based on the observations of numerous studies, it was discovered that strontium is capable of stimulating osteoblasts activities during the mineralization process and at the same time inhibiting osteoclasts. Likewise, fluorine was also found to assist in bone formation through similar stimulatory effect on osteoblast proliferation [45]. Interestingly, the amount of fluorine present in coral is 1.25–2.5 times greater that measured in human bone. Furthermore, magnesium is also beneficial during bone remodeling as it has been shown to increase the mechanical properties of newly formed bone [57]. Evidently, most of the elements found in bone can be located in different corals; however, they differ in their amounts and distribution.

3.4.2 Producing Bioactive Calcium Phosphates from Coral Exoskeletons

It has been reported that marine-derived calcium carbonate exoskeletons possess fast degradation rate that may not be suitable for long-term load bearing applications. On the other hand, it is worthy to mention that this property might potentially be useful in drug delivery applications where fast acting and short-term therapy is required. In order to overcome such limitations, the calcium carbonate exoskeleton of coral is converted to the more stable calcium phosphate structures and its derivatives such as hydroxyapatites, tri-calcium phosphates and their mixture as biphasic apatites, as applied in a number of studies [17, 58–60]. One of these conversion processes is commonly referred to as the hydrothermal exchange conversion strategy, which was developed in 1974 by Roy and Linnehan [61]. In simple terms, this process exchanges the carbonate component of the coral for phosphate to produce calcium phosphates and its derivatives using high temperatures (between 200 and 260 °C) for a period of 24–48 h.

The molar ratio of calcium to phosphate can be adjusted accordingly to yield different forms of calcium phosphates. In drug delivery or under certain circumstances in bone grafts, hydroxyapatite or tri-calcium phosphates are more ideal compared to other calcium phosphate compositions. In addition, tri-calcium phosphate compared to hydroxyapatite, has been widely examined and applied as bone grafts mainly due to its relatively faster dissolution rate [62]. Moreover, tri-calcium phosphate is also more ideal in drug delivery systems as a consequence of their appropriate controllable dissolution rate [63].

The hydrothermal conversion from calcium carbonate exoskeletons to tri-calcium phosphate would require a Ca/P molar ratio of 1.5. The time consumed in the conversion process is an important factor: a conversion time of less than 24 h would produce carbonated tri-calcium phosphate, while complete transformation will occur for a time period of over 48 h. More importantly, this also depends on the size of the material being converted.

In our studies with both coral and foraminifera shells, hydrothermal treatment with Parr reactor was used, the chemical conversion of these materials to hydroxyapatite

(microspheres) did not change the original structure, which makes it free to adsorb any drug compounds intended to be used and/or to permit the penetration of new bone cells into the micropores. Due to the pore architecture, these microspheres can dissolve within the physiological environment and supply calcium and phosphate ions and other ions and the drugs incorporated to the immediate bone structure [64, 65].

3.5 Marine Shells

3.5.1 Nacre

Natural biomaterials, specifically marine based materials, were widely used by physicians from ancient civilizations of India, China, Egypt and Central America and it was the ancient Mayans who discovered the unique property of nacre to heal seamlessly onto living human bone without causing harm [66].

Unlike any other biomaterial, nacre from the pearl oyster, *Pinctada maxima*, is able to induce osteogenesis and bone formation from latent osteoprogenitors along an endochondral pathway, consisting of a cartilage tissue intermediary phase.

Unexpectedly, the outer nacreous layer of a certain species of mollusc shell proved to be a valuable source of biomaterials for clinical applications, and in particular the tissue engineering of new bone. The scientific basis of fusion with bone was first discovered by Lopez et al. [66] and later by Lamghari et al. [67]. Under closer scrutiny, nacre was found to activate skeletal cells, induce bone formation and provide structural support in a human clinical trial [67].

Commonly known as the mother of pearl, nacre is featured in a large number of researches often in its powdered form. Nacre is the lustrous aragonitic inner layer found on molluscan shells in taxa such as mussels and abalone. Furthermore, nacre has both inorganic and organic components, with an organic shell matrix similar to bone. The organic shell matrix is composed of proteins, glycoproteins and polysaccharides, which serve as a template for calcium carbonate mineralization.

From a clinical perspective, the outer nacreous layer of certain species of mollusc shell is an unlikely and unexpected source of biomaterial for tissue engineering of new bone. In addition, nacre has been tested in human, sheep and rabbit models [67]. In human patients, fresh woven bone bonds itself throughout the nacre implant, augmented by the heightened activities of osteoblasts and osteoclasts (Fig. 3.4). Even though nacre is stably tolerated in vivo, its degradation and resorption are limited, and this could hinder its use within calcified tissue that requires rapid self-renewal [67].

The “water soluble matrix fraction” or WSM of nacre, despite the controversy concerning its definition according to nacre researchers, directly induces the formation of new bone [68, 69]. Moreover, WSM has also been shown to increase bone mineral density (BMD) in an ovariectomized mouse model of osteoporosis.

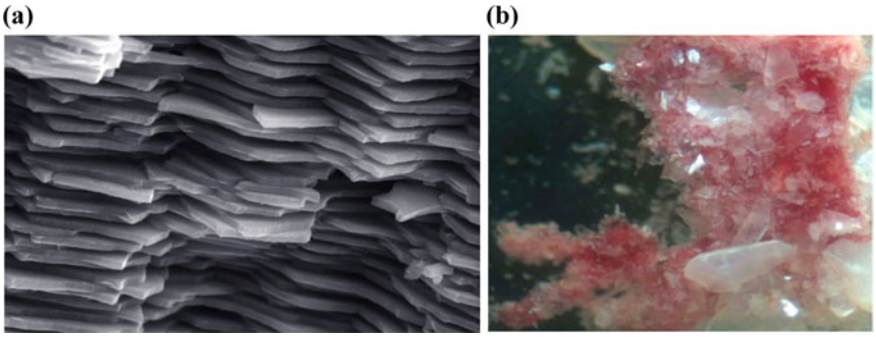

Fig. 3.4 SEM image of plate-like structure of nacre (**a**) and combined in vitro culture of hBMSC (**b**). Nacre chips of various sizes can clearly be seen. The cell mass is stained red for ALP secretion (a primary marker of bone formation)

Molecules from nacre matrix have been shown to decrease bone resorption by restricting osteoclast metabolism [70]. Based on the evidence available, it has been hypothesized that mobile signal transmitters involved in the biological control of mineralization dissolved into solution. These mobile signal transmitters, serving as an initiator and inhibitor of calcium carbonate crystallization at the growing front of mineralization, induce differentiation of surrounding latent osteoprogenitor cells [71].

The mechanism behind how nacre directly induces human cells to create new bone can be best explained using the notion that a "signaling" biomolecule is responsible for the regulation of cell-mediated biomineralization, which is common to both nacre and bone tissues of vertebrates. Consequently, these biomolecules must have been conserved by evolutionary selection pressures.

Mollusc shells are a fascinating model for understanding the complexities behind biomineralization such as the control and regulation of protein-mineral interactions. One of the prerequisites in the design of functional materials is to identify the proteins responsible. The so-called osteopromotive effects of nacre as measured by ALP expression are also commensurate with treatment with dexamethasone, at least in fibroblasts.

Size exclusion HPLC of the water-soluble matrix has uncovered protein fractions rich in glycine and alanine, with specific biochemical effects on human fibroblasts that modulate cell differentiation and proliferation [72]. Peptides are prevalent in the nacre matrix and certain fractions have been shown to give rise to specific responses from cultured osteoblast cells. Protein fractions with low molecular weight of less than 1 kDa for instance up-regulate ALP secretion whereas high molecular weight fraction reduces ALP secretion. Detailed sequencing of water-soluble proteins using proteomics offer enhanced characterization of nacre matrix proteins.

Nacre WSM was also shown to increase the secretion of a key inhibitor of apoptosis, cytoplasmic Bcl-2 and has an influence on rat calvarial osteoblast maintenance and survival. Low molecular weight fractions were recently found to increase

expression of collagen type I and the osteogenic associated mRNA expression of osteopontin and Runx-2 [69].

Further detailed characterization of the bioactive low molecular weight molecules has led to the identification of 110 molecules in the 100–70 Da range comprising of glycine-enriched peptides with structural similarities and high affinities for each other. A highly defined matrix protein with a 10 kDa size named as p10 has specifically demonstrated an increase in human fibroblast cell ALP expression lending greater hope that the osteogenic signal molecules can be isolated in their vital functional form [73]. A soluble p60 protein conglomerate extracted from decalcified nacre possesses sufficient bioactivity on 3T3 and MSC to induce the secretion of mineral nodules. Some of the specific biomolecular mechanisms and associations between the signal molecules and cellular processes are being gradually uncovered [20].

Nacre WSM can be broken down into a number of fractions containing amino acids of varying size and composition. In addition, a number of proteins have been identified within the nacre of different species and many of which are thought to have roles in regulating bone tissue. One such example is the oyster *Crassostrea gigas* where four unique proteins were identified from proteomic nacre analysis and believed to assist in shell mineralization, with structures homologous to endogenous human proteins and with roles in osteogenesis. In addition to P10 and P60, novel single proteins, such as PFMG3 have also been identified; all sourced from the pearl oyster *Pinctada fucata*. It is also interesting to note that these proteins were found to enhance crystallization of calcium carbonate in vitro.

Some researchers in biomineralization questions the fact that nacre proteins are the primary cause of osteoinduction even though it was believed that nacre promotes osteointegration. Nacre failed to stimulate an in vivo osteogenic response as discovered in a study by Liao et al. [74], although bone-to-nacre apposition and bonding did occur directly. The author suggested that nacre provided a favorable surface chemistry, rich in phosphorous, favourable to osteoclast and osteoblast recruitment, attachment and matrix synthesis [74]. In an in vivo ectopic bone environment, surface modified nacre was found not to be osseoinductive within demineralized bone matrix, but its integration and fusion with bone was better than non-nacre controls.

The relationship between interfacial properties and biocompatibility of nacre was examined in a study by Kim et al., and in particular its unique bone-bonding capability [75]. They concluded the reason behind the excellent bonding between nacre and bone tissue was the presence of an organic matrix that creates a favorable surface charge for optimal biological associations. The creation of a new interfacial microenvironment was hypothesized once the organic matrix of nacre is implanted that generates many functional associations with the surrounding tissue. Ultimately, this leads to an enhancement in bonding with bone in comparison to the implantation of bioceramic implants that does not contain any organic matrix.

The WSM was identified to be responsible in the formation of this HAp layer and the augmented cell responses [68]. Taken altogether, nacre provides an appropriate tissue-compatible physical platform that show unique peptides that initiate and drive bone formation.

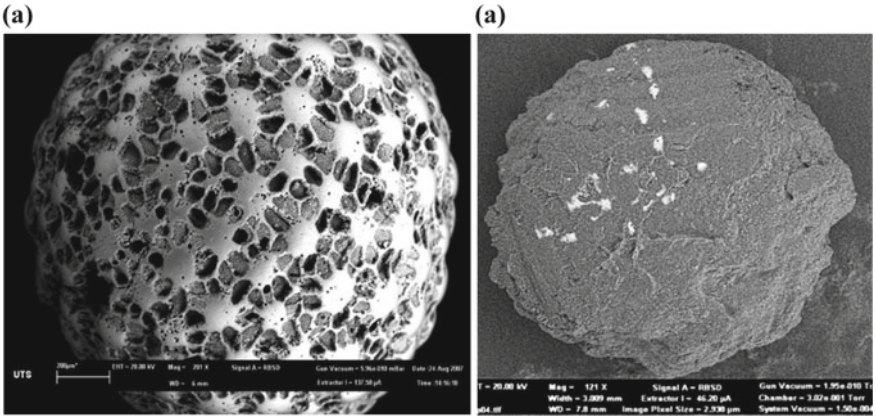

Fig. 3.5 SEM image of a Foraminifera (Baculogypsina sphaerulata) microsphere (**a**) and the potential of macrospheres to anchor and transport adherent stem cells fit for transplantation. **b** SEM image of β-TCP macrosphere coated in adipocyte-derived stem cells in 3-dimensional pellet culture

Furthermore, due to its organic content and plate like design, nacre is mechanically tough, non-immunogenic and rapidly biodegradable, without eliciting detrimental physiological effects. These characteristics of nacre provides a unique substrate for delivery of a functional (possibly osteopromotive) agent to sites of bone loss in quantities that lead to rapid bone repair and regeneration.

3.5.2 Foraminafera Shells (Coral Sand)

In our research, specific marine shells were collected and they were identified as spherical fossilized shells called Floresianus (Foraminafera) from coral beach sand of Great Barrier Reef, Australia. The samples were intact, lacked spines, and measured 0.5–1.5 mm in diameter (Fig. 3.5). These coral sand shells or more appropriately microspheres possess unique fenestrated structures that have evolved to circulate seawater and collect light for the mutual benefit of symbiotic algal cells that reside inside the shell.

Microcomputed tomography (μ-CT) and scanning electron microscopy (SEM) imaging confirmed that these shells were internally permeated by a 3-D network of microscopic interconnected channels measuring 1–10 μm in diameter. Moreover, nano- and meso-pores are contained between the micropores surface area.

It was necessary to convert these microsphere shells into more stable, highly crystalline β-tricalcium phosphate (β-TCP) and hydroxyapatite (HAp) using the hydrothermal conversion methods developed earlier [65, 76]. The converted microspheres were further coated with stem cells for orthopedic and maxillofacial appli-

cations [77]. Additionally, these foraminifera microspheres were used as bioactive bone grafts and drug delivery devices after conversion to calcium phosphate [78].

3.6 Marine Sponges

At present, collagenous marine sponges are extensively exploited for novel biological compounds as potential treatments for cancer tumors, leukemia and inflammation. Marine sponges are also a source of collagen for cosmetics [79] and dermatological preparations [80]. Approximately 50% of all marine-derived materials are sourced from a wide spectrum of marine sponges. Collagenous marine sponge skeletons are incredibly soft, but strong, highly absorbent, elastic and resistant to bacterial attack. Such properties make them highly suited for clinical applications. As a result, collagenous marine sponges possess the potential to be used in the development of clinically relevant scaffolds for a range of tissue engineering applications such as bone, cartilage, fat connective, liver and kidney.

The fiber-bonded meshwork provides conduits for cell guidance together with spaces for rapid tissue infiltration and in filling. It has been discovered that the collagenous composition of the fibers promotes attachment of all human cell. Their high wettability and adsorption of growth factors onto the collagen fibers can be explained by their unique layered ultrastructure that allows for their infusion into attached cells and promote their activities [34].

In vivo observation revealed extensive (in which the entire sponge implant is completely filled) and well-developed tissue formation within four weeks with the structure and quality of tissue being equivalent to immature bone (Fig. 3.6a–c) and neocartilage. Mineralization is also evident in these tissues following positive staining with alizarin red staining. Collagenous marine sponges with large (1,000 nm) voids and innate semi-rigidity supported the growth of soft human adipose tissue (Fig. 3.6d). According to live/dead cell staining and SEM analysis, it was concluded that marine sponge matrices can support extensive cell adhesion and proliferation.

Furthermore, sponge scaffolds were able of retaining their original shape after three weeks of subcutaneous implantation within immuno-compromised mice. Upon dissection, a typical vascular supply network was discovered and reported by Roberts et al. that has developed into the soft tissue [81]. These results demonstrate the feasibility of utilizing marine sponge biomatrices in soft tissue engineering and tissue regeneration. The addition of biogenic additives has been found to effectively modulate the size, morphology, and architecture of these sponge microspheres. One such example is the incorporation of nacre proteins to chitin nanofibrils in which mineralization was modulated very effectively (Fig. 3.7).

Marine sponges possess the most primitive form of extant tissue (600 million years old) but share much in common with multi-cellular tissues which have apparently conserved many features evolved by these first multi-cellular organisms. Morphological and biochemical similarities exist between marine sponge and vertebrate extra cellular matrix (ECM) alluding to fundamental rules of organization evolved first

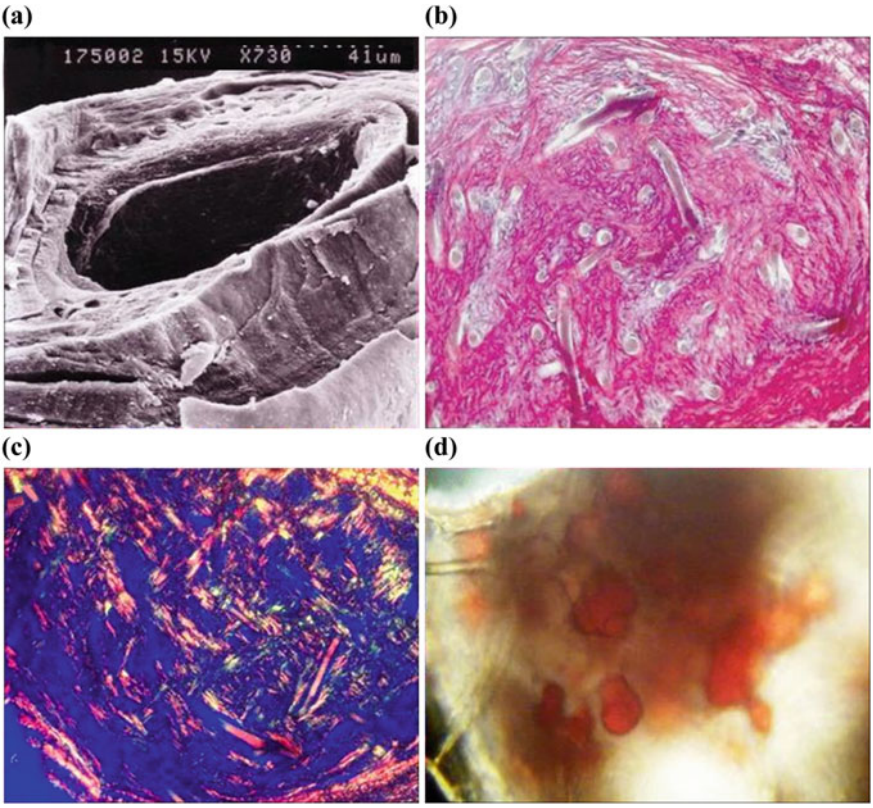

Fig. 3.6 Marine sponge scaffold for tissue engineering of soft and hard human tissues. **a** SEM ultrastructure of marine sponge fiber; **b** histological section through marine sponge filled with foetal cell derived tissue following implantation for 28 days; **c** bi-refringency of developed tissue within marine sponge showing highly ordered organization of collagen fibers; **d** in-situ staining of lipid droplets with Oil red-O staining

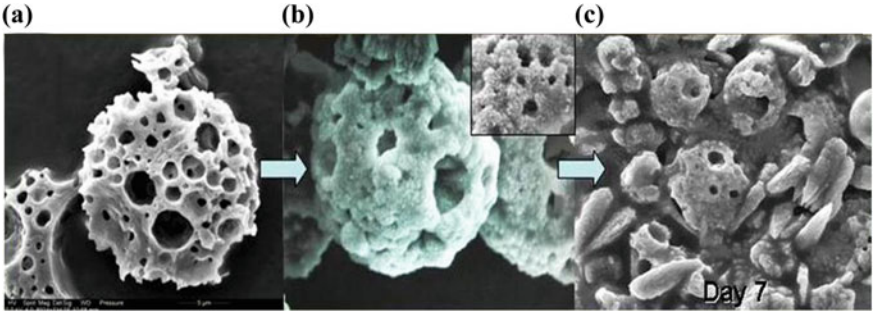

Fig. 3.7 SEM images of vaterite microsphere degradation over 7 days. **a** Fully formed vaterite microsphere at day 0; **b** partially decomposing vaterite spheres at 4 days; **c** significant decomposition and phase change of spheres into rhombohedral crystals at day 7

by marine sponges. Three collagen types have so far been identified from marine sponges.

In a similar fashion to vertebrates, collagen fibrils in all sponges are secreted in bundles that are composed of thin fibrils 22 nm in diameter with highly ordered periodic banding. Moreover, the amino acid sequences and genome organization is also similar in comparison to vertebrate collagens despite the fact that the collagen ultrastructure is relatively simple. In addition, collagen fibrils are closely associated with proteoglycans, which in mammalian tissue sets the form and shape at long-range scales.

Fibronectin, dermatopontin and tenascin polypeptides are also found in marine sponge collagen fibers and cross-react with antibodies raised against vertebrate analogues highlighting their common origins. Some sponge species possess an analogue of type IV collagen found in vertebrate basement membrane collagens [82]. The organization of collagen fibrils is analogous to collagen type XIII that makes cells adhere to surfaces. It is with these properties (fibronectin and cell adherent collagens) that collagenous marine sponges represent a significant potential for future development as bioactive tissue engineering scaffolds.

Similar to artificial coral, the exact conditions required to cultivate marine sponges at a large enough scale viable for commercial applications are being explored by a number of researchers. Some have established aquatic pilot farms for the cultivation of selected bath sponge species. Marine sponges are sufficiently adaptable for commercial scale production. Another intention behind the cultivation of marine sponges is to extract medically important secondary metabolites in much larger quantities than is possible from collections made using conventional bio-prospecting.

Marine sponges are also being investigated in cutting edge research and, in particular, in the design and fabrication of new advanced functional silica materials. This leads to the production of more efficient conductors of light and therefore as models for future fiber optics. The superb and optimized structural design of silica marine sponges has been hypothesized to provide useful lessons in the construction of synthetic frameworks with ultimate strength using minimal starting materials [40].

Collagenous marine sponges fulfil the potential of a clinically relevant scaffold for a range of tissues including bone, and cartilage.

3.7 Supply

During the development of marine-based biopharmaceutics and bone grafts, factors such as purity, consistent supply, environmental concerns, and their long-term protection are some of the key issues or limitations that need to be addressed. Another major concern is specifically related to the use of marine structures from endangered species. Over the past two decades, many research groups have implemented successfully marine materials from either collecting fossilized marine structure instead of using live ones or grown them artificially.

A rigorous process must first be carried out before any marine material can be used as a bone graft or drug delivery device or carrier material. This ensures the quality of the material is controlled from collection to fabrication and to its final application.

Due to an increase in sensitivity with modern screen techniques and within the limits of detection, studies can be carried out to ensure that no traces of organic materials or foreign entities can be found and that the material is of the highest quality and purity. These studies can include optical, radiographic, chromatographic, spectrophotometric and biocompatibility analyses. Unless the study specifically requires the presence of proteins and organic matter, any residual organic constituents are removed prior to sterilization of the calcium carbonate material by immersing in solution of sodium hypochlorite for at least 1 h and then drying at about 100 °C followed by ultrasound treatment. Sterilization of converted hydroxyapatite is usually carried out with gamma irradiation.

3.8 Concluding Remarks

Although we are producing incredibly intricate structures with using 3-D printing technology, natural biomaterials possess enviable properties such as complexity, sophistication and miniaturization that are not yet possible to fabricate fully in the laboratory. However, we are gradually inventing ways of replicating nature to produce similar levels of sophistication albeit to a limited extent.

Presently, we are only able to recreate microscopic structures with some level of biomimetic detail. This has been particularly true for the replication of marine structures. One versatile approach has been to use biological microstructures as templates for the reproduction of inorganic structures with identical features.

Current strategy has been to synthetically replicate the structures of selected inorganic biomatrices. They have clear significance to the production of replacements for calcified tissues. This is achieved by using techniques in biomineral-inspired materials chemistry. The concept is based on the consecutive developmental pathway of systems that nature employs to make skeletons from molecules into macroscopic structures.

In nature, all the necessary building materials are supplied in a continuous manner in an effort to keep pace with the progress of construction. It begins with supramolecular pre-organization, interfacial recognition, vectorial regulation lending to multilevel processing. These processes are developed within confined reaction spaces directed in their formation by the templates themselves. The continual multiplication of these assemblies builds-up into the emergence of morphology and macro-scale biomimetic forms.

These bio-replication strategies and approaches are being pursued with cellular and extracellular matrices (ECM) inputs and include factors such as mineralization of reverse microemulsions, mineralization of bi-liquid foams, mineralization of bicontinuous microemulsions, template mediated biomineralization of organic

biomatrices. This approach has yielded clinically relevant end-results. Biomimetic microspheres created within self-organizing microemulsions were routinely synthesized and served as highly functional paradigms for the localized delivery of growth factors and genes to primary human cells. These unique particles were also proficient at producing osteoid and neo-cartilage.

In addition to above studies, new research is taking place with natural materials such as marine structures and the production of new devices which adapt to their environment. This enables mankind to synthesize an exciting array of self-responsive structures and materials for regenerative medicine.

Biomaterials in nature are produced with immaculate resource and energy efficiency using common, readily available substrates through self-assembly into highly organized hierarchies. Functional structures optimized to their environment are produced in this way. Most marine structures are made of calcium carbonates which can be easily converted to calcium phosphates with various bioactivity.

This provides mankind an opportunity of creating structures with intricate architectures and shapes which are customized to their functions without the risk of failure or destruction. From a clinical perspective, they can be utilized to assist tissue regeneration, the delivery of minerals and drugs, and to repair soft and hard tissues.

Previously, we have revealed how biomimetic approaches can yield promising outcomes for application in tissue engineering of skeletal tissues. Our research is a part of an ongoing effort towards the design of clinically relevant scaffolds for regenerative medicine using a unique set of self-organizing hierarchical structures designed and synthesized according to biological principles of design. Of vital importance is their bioactivity which promotes early and easy osseointegration.

There is a clear and present need for better tissue engineering scaffolds that possess more natural bio-responsive environments conducive to guiding the natural processes of regeneration which can be highly intricate and dynamic in space and time. The issue of future implants rests on in situ adaptation of synthetic or natural materials within its biological environment. Nature supplies both the scaffolding, relevant biogenic material and design. Cells alter their environment as they proliferate and differentiate. Future advanced biomimetic scaffolds must be able to adapt to these changes and meet the ever-changing needs of developing tissues.

The future of implants depends on the designs that possesses artificial intelligence to overcome this biological challenge. We contend that there needs to be a gradual change in the scaffold environments that are responsive whereby the synthesized biomatrix evolves in real-time to meet the demands and optimizations of adaptive growth and regeneration of human tissues.

Synthetic materials with characteristics and function similar to those found in biological materials are currently being developed and manufactured without direct cell guidance and under no genetic control. Our next challenge will be to grow or print (using 3-D printing techniques) materials and designs using cells as well as promoting their regulation during material fabrications of implants and functional organs.

Moreover, materials with the capacity to self-repair, self-respond, and self-actuate are also being pursued for applications in construction, defense, and in engineering

industries. In addition, the need to develop biomaterials which continuously adapt their growth and composition and ultimately their function as well as having the capacity to self-repair when the rate of adaption is too slow or fails completely in a dynamically changing environment is also being examined.

Advanced functional materials with the above-mentioned characteristics would see enormous benefits in engineering and bio-pharmaceutical industries. At a time when fewer resources are being consumed during the manufacturing process, utilizing sustainable resources and using less energy during the design and fabrication of a new technology is of vital significance.

As previously mentioned, the incorporation of natural and converted bioactive natural materials, nanofabrication techniques, and 3-D adaptive printing methods using biological principles of assembly and design is still in its infancy. The use of this bio-inspired nanofabrication and 3-D printing technique for tissue engineering is a unique approach that has huge potential to improve the design of scaffolds matching to the physico-chemical environment in which they are intended with an ability to micro-evolve. Such revolutionary technology will also create benefit in science and engineering and its application.

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Besim Ben-Nissan Prof. Ben-Nissan has B.Eng. in Metallurgical Engineering (ITU), M.Sc. degree in Ceramic Engineering and Ph.D. in Mechanical and Biomedical Engineering both from the UNSW Australia.

During in his formative years Prof. Ben-Nissan worked on Titanium and its alloys and Magnesium alloy development and casting technologies and their properties for both engineering and medical applications. Over the last four decades, Professor Ben-Nissan has worked and contributed to the biomedical materials, implant design, production and analysis of various advanced ceramics, nano-coated sol-gel developed thin films, coated orthopedic and dental implants, anti-microbial slow drug delivery devices and methods, marine structures for clinical applications, biomechanics and finite element analysis of medical materials and engineering structures.

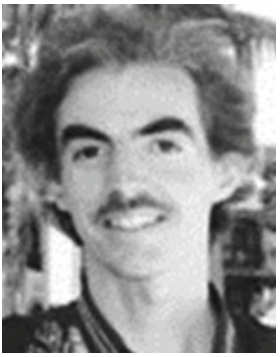
He has successfully developed materials for implant technology such as ceramic knee prosthesis, calcium phosphate based bioactive materials, bone graft production and bio-composites, and conducted research on biomechanics and modelling such as jaw bone, knee and hip joints, reliability and implant design modular zirconia ceramic knee prosthesis, femoral head and taper stresses and artificial ocular implants and bionic eye and recently on 3D printing of bioceramics and metallic implants and anti-microbial multifunctional coatings for drug delivery which are supported by the European Commission and the Australian Academy of Science research grants.

Since year 2000 he has published over 200 fully refereed papers in journals, and a book and 43 book chapters. He edited a book on Calcium phosphates and working on a second one on the use of Marine Structures in the Biomedical field. He is the editor of the Journal of the Australasian Ceramic Society. He was awarded by the Australian Ceramic Society's prestigious award for his contributions to the "Ceramics Research & Development and Education in Australia". For his research on multifunctional nanocoatings he also received "The Future Materials Award".

Andy H. Choi Dr. Andy Choi is an early career researcher who received his Ph.D. from the University of Technology Sydney (UTS) in Australia in 2004 on the use of computer modelling and simulation known as finite element analysis (FEA) to examine the biomechanical behavior of implants installed into a human mandible. After completing his Ph.D., he expanded his research focus from FEA to sol-gel synthesis of multifunctional calcium phosphate nano coatings and nano composite coatings for dental and biomedical applications.

In late 2010, Dr. Choi was successfully awarded the internationally competitive Endeavour Australia Cheung Kong Research Fellowship Award and undertook post-doctoral training at the Faculty of Dentistry of the University of Hong Kong focusing on the application of FEA in dentistry and the development of calcium phosphate nano-bioceramics.

He is served as an associate editor for the Journal of the Australian Ceramic Society and on the editorial boards for a number of dentistry, nanotechnology, and orthopedics journals. To date, Dr. Choi has authored over 50 publications including 3 books and 26 book chapters on calcium phosphate, nano-biomaterial coatings, sol-gel technology, marine structures, drug delivery, tissue engineering, and finite element analysis in nanomedicine and dentistry.



David W. Green Dr. Greem is working at the moment the bioengineering interface between physical, chemical and biological phenomena; He is attempting integration of non-living matter with living matter for manufacture of bioinspired systems. Consequently, to revitalize cells and tissues into novel regenerative therapies. Accordingly, he is guided by biomimetics and bioinspiration philosophies to create new innovations for healthcare. Presently, Dr. Green is focused on biomimetic development of anti-biofilm materials and stem cell niches.

Part I
Marine Sources for Biomaterials

Chapter 4
Nanobiomaterials for Bone Tissue Engineering

Baboucarr Lowe, Fernando Guastaldi, Max-Laurin Müller, Fredrick Gootkind, Maria J. Troulis and Qingsong Ye

Abstract Biomaterials with functional properties are used to fabricate scaffolds for bone tissue engineering. Several of these materials can be derived from nature, processed and transformed into regenerative scaffolds and/or artificial matrices for applications in bone tissue repair or regeneration. In this chapter, we discuss the basic biology of bone development and the utilization of chitosan, hydroxyapatite and diatoms for BTE. The regenerative properties of Chitosan are desirable due to its close proximity with glycosaminoglycan—an extracellular matrix polysaccharide, which interacts with collagen fibers. Nano-hydroxyapatite is an inorganic component of natural bone matrix with osteoinductive properties. Diatoms are important source of biogenic silica and their high surface area, as well as nanoscopic pore structure make them desirable for delivery of biomolecules and reinforcing structural functions of three-dimensional scaffold matrices. Additionally, we discussed the methods used to fabricate the scaffolds for bone repair.

Keywords Nanobiomaterials · Bone tissue engineering · Chitosan · Nano-hydroxyapatite · Diatoms

4.1 Introduction

Bone graft transplantation is one of the common surgical procedures performed [1]. It is estimated that every year at least more than a couple of million bone graft procedures are performed worldwide, making bone one of the most transplanted tissues [2]. In the United States alone, this number is more than half a million procedures each year [3]. While the body's own repair mechanism is often sufficient to repair

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simple fractures, larger, more extensive or more severe defects often require a graft in order to restore structural integrity and full function.

The use of autogenous bone remains the gold standard for bone repair; however, the harvesting of autografts is also associated with clinical complications such as bleeding, infection, fracture and sometimes severe pain at the donor site [2, 4–9].

Donor bone (allograft) are also used; on the other hand, given the risk of infections such as Hepatitis C, HIV etc., allografts are usually processed prior to transplantation to minimize the risk of disease transmission, which reduces the biological activity of the tissue [2]. The major processing methods used are demineralization or freezing. Both process results in death of the host cells [10]. Due to these challenges the application of synthetic materials to replace damaged bone tissue have gained considerable research attention.

Nature is an excellent source for many biomaterials, compound, and other biological as well as chemical products. Biomaterials are used for the fabrication of artificial matrices to serve as support base for new bone tissue in addition to promoting the development of maturing of bone cells. There are three major components that must be considered in bone tissue engineering: (1) the material used in and design of the scaffold; (2) the cells that will be seeded into the scaffold; and (3) the factors that affect the growth and integration of the scaffold into the skeletal matrix [3].

From a clinical perspective, the reconstruction of bone defect is faced with several other challenges associated with defect size, anatomical area, and mechanical load. By using bone tissue engineering approach and/or techniques, biomaterials are designed and synthesized in an effort to increase the growth affinity of cells, improve integration and to promote the growth and deposition of new bone tissues [11]. A number of materials have been investigated for this purpose. Of vital importance is the introduction of nanomaterials as they have generated some special attention due to their specific properties [12]. These properties will be discussed in the latter sections of this chapter.

4.2 Bone Biology

The internal structure of the human body consists of bone. This biological material is giving the human body a solid and physically stable structure. Typically, bone has a high resistance against pressure and tension forces, whereas it is minimally flexible [13]. Thus, bone has a supportive function. Moreover, the protective function of bone should be mentioned as well. It protects the body inner organs, by covering them. For example, the thorax protects the inner organs and the skull protects the brain tissue. Bone is a huge reservoir of calcium and phosphorus minerals which play significant roles in bone metabolism [14, 15].

Although the bone composition varies in different bone types and stages of development, all human bones consist of the same basic elements. Natural bone matrix is made of organic and inorganic constituents [16]. The organic part is mostly formed

by collagen (especially Type I), glycoproteins and proteoglycans. The inorganic part is hydroxyapatite and ions, like magnesium and fluoride [17, 18].

Another important part of the bone is the bone membrane that functions as a thin protective layer at the internal and external surface of the bone. The periosteum is a connective tissue layer on the outer surface, having two sublayers: the cambium and the fibrous layer. On the other hand, there is the endosteum, a bone membrane on internal surface of the bone. It also covers the Haversian canals, the Volkmann's canals and the canaliculi [19].

Another important part is the bone marrow. There is a distinction between red- and yellow bone marrow cells, which have different functions in the human body. The red bone marrow plays an important role in hematopoiesis, whereas yellow bone marrow cells are inactive. There can occur big deposits of adipocytes.

At the cellular level, osteoblasts, osteocytes, bone lining cells and osteoclasts make up bone matrix [18, 20]. These cells (osteoblast, osteocytes and bone lining cells) can developed from mesenchymal stem cells and Osteoclasts via fusion of monocyte precursor cells [18].

Osteoblasts function as bone building cells [21]. They differentiate from osteoprogenitor cells [20] and are stimulated by transforming growth factor-beta (TGF-β) and bone morphogenic proteins (BMP). Moreover, osteoblasts have parathyroid receptors, therefore they can be influenced by thyroid stimulating hormone (TSH) [22]. They produce osteoid, an organic bone matrix, which is deposed in layers on the mineralized matrix. Also, osteoblasts control osteoid mineralization, the formation of hydroxyapatite, as well as bone remodeling, by acting as a counterbalance to the destructing osteoclasts. These cells are very concentrated along the surface lining of the bone, at the periosteum and endosteum [18].

Another important group of bone cell is osteocytes. These are osteoblasts which got trapped by new osteoid layers on top. The role of osteocytes is not fully understood. However, it could be observed, if osteocytes are missing, their associated bone matrix is cleared by osteoclasts. Therefore, osteocytes appear to be involved in the maintenance and remodeling of the surrounding bone matrix [23, 24]. Bone remodeling involves a series of coordinated processes, which can be divided into three major steps: resorption, reversal and formation as shown in Fig. 4.1.

Furthermore, the osteocytes lie in bone cavities and are connected to surrounding cells by gap junctions in bone tubules [20]. These bone canals perform a number of functions: it is possible that these canals can establish communications between cells via gap junctions, while on the other hand, the osteocytes can be supplied with the necessary building materials, such as ions, for further mineralization of the bone [17, 25].

Furthermore, the function of the bone lining cells is still obscure, however, they are shown to prevent the direct interaction of osteoclast with native bone matrix, when resorption is not needed, and the matrix needs protection. The bone lining cells also take part in differentiation of osteoclasts [25], by producing osteoprotegerin (OPG) [26] receptor activation of nuclear factor kappa-B ligand (RANKL) [27, 28].

Last, but not least, osteoclasts are cells, which dissolve the bone matrix. The main function is bone remodeling and resorption. These cells are multinucleated

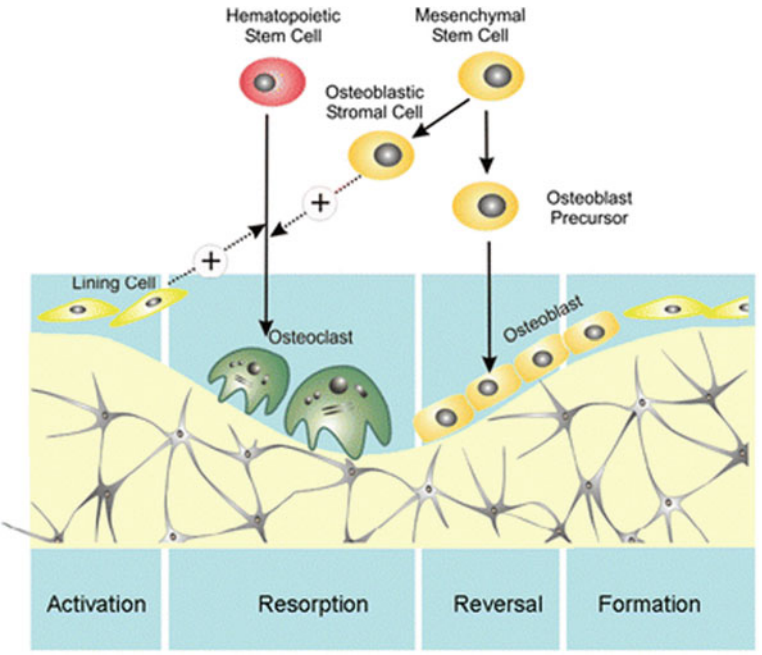

Fig. 4.1 The sequential events associated with bone remodeling, showing the phases of activation, resorption, reversal and formation

phagocytes, which degrade mineralized bone. There are two ways how osteoclasts, can dissolve bone structure. First, bone minerals are dissolved by reduced pH between the space separating the osteoclasts and bone matrix. This pH is maintained by active proton transport. Alternatively, the osteoclasts release proteolytic enzymes that dissolve the collagenous bone matrix. Subsequently, the released collagen fragments are phagocytosed. Osteoclastic resorption is very effective compared to osteoblastic bone matrix production. An osteoclast breaks down the same amount of bone that 100 osteoblasts build up during the same time. Osteoclasts stem from monocyte precursor cells influenced by Microphage Colony Stimulating Factor (M-CSF) and the RANK-Ligand [29]. These factors also influence activation of the transcription factors [30, 31], as well as the gene expression [32].

Ossification is the formation of bone tissue during growth after fractures or pathological events. Osteogenesis refers to the formation of an individual bone. Bone can arise in a variety of ways during the development process. They can be produced through intramembranous osteogenesis from connective tissues or created by endochondral ossification using cartilage. The formation of bone through intramembranous osteogenesis may account for the growth of the vault of the skull, most of the mandible and clavicle. On the other hand, bone formed in the axial and appendicular skeleton, parts of the mandible and the base of the skull is through endochondral ossification [18].

During the process of intramembranous ossification, bone is developed directly out of connective tissue. At the early stages of development, mesenchymal stem cells differentiate in the center of ossification. This is followed by osteoblasts producing osteoid, which leads to a development of osteocytes after the mineralization of osteoid. Ultimately, this leads to the formation of a bone segment. Primary trabecular bones are formed by merging a number of bone segments together. Lamellar bones are created from woven bone using the simultaneous, construction and reconstruction of the bone during this process.

In contrast, there is endochondral ossification in which bone is formed indirectly from mesenchymal stem cells and differentiates into chondroblasts. The chondroblasts form a cartilaginous model, and finally, the cartilage turns into bone. Most human bones are developed through endochondral ossification [18].

4.3 Application of Chitosan Nanocomposite in Tissue Engineering

Chitin is primarily the main source of Chitosan. Chitin which is a highly abundant material and can be easily isolated from the exoskeleton of crustaceans [33]. It can also be isolated from corals [34] and mushrooms [35]. Chitosan is derived from the deactylation of chitin. This major chemical hydrolysis method forms a pathway towards deriving the chitosan product. This method involves four key steps, namely:

1. Demineralization;
2. Deproteinization;
3. Decoloration; and
4. Deacetylation.

Chitosan has a linear chemical backbone with randomly distributed subunits made of (*N*-acetyl-D-glucosamine and (D-glucosamine) linked by β (1 → 4). The degree of deactylation directly corresponds to the sum molar ratio of the D-glucosamine to the *N*-acetyl-D-glucosamine present [36–38].

The fabrication a chitosan-based nanocomposite can be achieved by either combining chitosan with another nano-material entity or by transforming chitosan into chitosan nanoparticles. The role of the nanomaterial in either fabrication direction provides functional therapeutic effect associated with their structural and chemical properties. Chitosan can be transformed into nanoparticles using the following methods:

1. **Ionic Gelation of Chitosan with Sodium Tripolyphosphate (STP)**: Briefly, the chitosan 0.1% (w/v) is dissolved in acetic acid (0.1% v/v) and stirred and filtered (pore size 0.22 μm). The chitosan solution is then cross-linked with STP using a Pediatric set under 700 rpm magnetic stirrer. The product is finally centrifuged, pellets re-suspended in water, and sonicated at 80% pulser ratio for 100 s at 4 °C.

This process is repeated three times before the final product is precipitated and lyophilized [39].

2. **Acid/Base Titration Method**: In this method, 500 mg of 85% deacetylated chitosan was initially dissolved in 1% acetic acid, and then stirred at 100 rpm for 5 min. The homogeneous solution was further sonicated and titrated by using sodium hydroxide (NaOH) or hydrochloric acid (HCl) solution and pH stabilized to 5. The solution was finally filtered using 0.2 μm mesh [40].
3. **Microreactor Method**: This approach utilizes a microreactor to produce monodisperse nanoparticles. Syringes containing chitosan (0.25, 0.5, 0.75 mg/ml) and TPP (1.6 mg/ml) of equal volume (2.5 ml) are mounted to a syringe pump, connected to the microreactor inlet via tubing, and maintained at a follow rate of 100–500 μL/min.

Chitosan is an ideal material for bone tissue engineering due its close resemblance to glucosaminoglycans, which a component of the extracellular matrix that interact with collagen fibers and thus enhances cell adhesion [41]. Chitosan can be transformed into various structural geometries, as in fibers, films, sponges for regenerative applicability [41]. It can also be turned into three-dimensional (3D) lyophilized scaffolds and combined with ceramic materials to improve its osteoinductive properties [42].

In addition to its remarkable biocompatibility, chitosan can form high porous networks, which provides an ideal internal architecture for the attachment of cells, proliferation [43]. These events are showed in Fig. 4.2.

In this study, nano-hydroxyapatite (n-HA) was synthesized using calcium and phosphate precursors via a hydrothermal method. To fabricate the scaffold nanocomposite, a slurry of n-HA particles was initially mixed in alginate solution, followed by addition of chitosan solution. After mixing, the final mixture was casted into plate and lyophilized. The combination of chitosan with alginate forms a polyelectrolyte

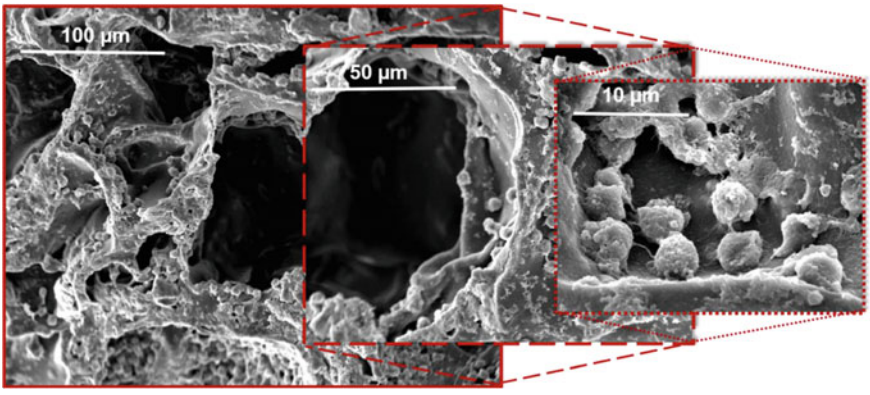

Fig. 4.2 Scanning electron microscopic image showing cell adhesion in a chitosan scaffold matrix. Reproduced with permission from [44]

complex and improves the mechanical and structural stabilities of the produced composite. The results demonstrated uniform pore networks as well as an increase in both compressive and elastic moduli of the composite. Furthermore, observations made during the in vitro assessment indicated an increase in osteoblastic activity and mineral content [45].

Others researchers have fabricated a hybrid delivery system using calcium phosphate cements loaded with bone morphogenic protein-2 mixed with sulphated chitosan for bone tissue regeneration. This hybrid system was compared to calcium deficient hydroxyapatite-loaded bone morphogenic protein-2 and calcium deficient hydroxyapatite in rat cranial defects. The results of the study showed that the percentage of newly formed bone was much higher in hybrid scaffold compared to other groups. The sulphated chitosan also enhanced the localized release of the BMP-2 for bone tissue engineering [46].

The application of chitosan for guided bone regeneration has attracted much attention due to its anti-microbial properties. Chitosan nanofibers can be generated by initially mixing chitosan (1.5% v/v) with 1,1,1,3,3,3-hexafluoroi-sopropanol (HFIP) and methylene chloride. Non-interwoven membrane can be produced using a voltage of 20 kV through a feeding rate of 0.5 ml/min and maintaining a distance of 15 cm from the collector, followed by vacuum drying at −100 °C. The synthesized membrane has a diameter range of 200 nm and median pore size of 5 μm with great than 80% porosity [47].

To improve the properties of a membrane for guided bone regeneration, other materials with bioactive ions are strategically combined using hybridization mechanisms. For examples, in a study by Lee et al. [48], they synthesized a guided bone regeneration membrane by combining chitosan with silica xerogel hybridized via sol-gel. Due to the organic/polymeric phase of chitosan, the incorporated silica xerogel produced a membrane with flexible behavior, which is a typical characteristic of any membrane. In addition, the produced membrane possesses bioactive properties suitable for the delivery of other molecules. The bone regenerative properties of the membrane were significantly higher and strongly associated with apatite nucleation capacity, cellular response, and mechanical properties of the hybrid [48].

4.4 Application of Nanohydroxyapatite-Based Nanocomposite for Bone Tissue Engineering

The application of nano-hydroxyapatite in regenerative medicine has attracted much interest. Nano-hydroxyapatite particles of size range between 50 and 100 nm have considerably higher surface area that enhances the biding of proteins [49–51].

Hydroxyapatite has long been studied and this is due to its biocompatibility and for being a close constituent of natural bone and teeth making up about (60–70%) and 90% of bone and enamel, respectively. Hydroxyapatite is also derived from marine resource such as fish bone [52–56] using various other methods including thermal

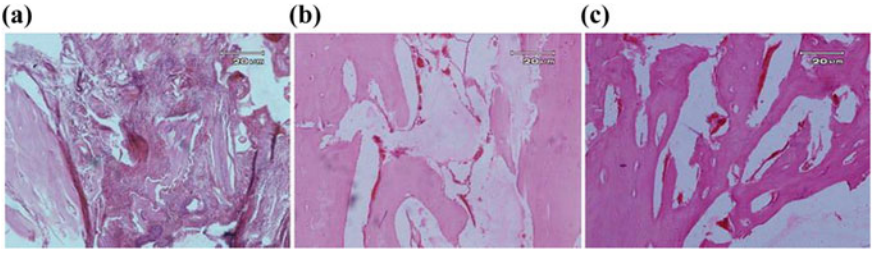

Fig. 4.3 Hematoxylin and eosin staining showing trabecular bone regeneration in (a, × 10 mag.) hydroxyapatite–DCFGP group (b, × 10 mag.) and DCFGP group (c, × 10 mag.) in 8 weeks old defect. Reproduced with permission from [62]

calcination, alkaline hydrolysis [54, 57, 58]. Other sources also include fish scales [59, 60] and sea urchins [61]. Furthermore, nano-hydroxyapatite isolated from natural sources (like those mentioned above) have shown promising outcomes in bone tissue engineering applications as revealed in Fig. 4.3.

For regenerative applicability, several structural modifications can be achieved during the synthesis of nano-hydroxyapatite. For example, HAp can be prepared in the form of dense or porous ceramic powder, coated to the surface layer of implants to improve osteointegration and to promote bone regeneration [63, 64].

More importantly, nano-hydroxyapatite has been widely used in oral and maxillofacial surgery. This material possesses excellent osteoinductive capacity and improves bone-to-implant integration. The applications of nano-hydroxyapatite in the field of oral implantology have been well documented [50, 51, 65]. In addition, collagen membranes associated with nano-hydroxyapatite have been applied in the form of bone defect filler and have shown promising improvements in periodontal indices [49].

From a clinical perspective, granular form of HAp is currently used for periodontal bone defect reconstruction and as bone filler following cystectomy, apicoectomy, loss of dental implants, as well as increasing the thickness of atrophic alveolar ridges. Other forms, such as blocks, have been used to reconstruct bone defects after trauma, osteotomies and for the stabilization of fractures, reconstruction of facial skeleton, replacement of parts of orbital and maxillary bone. Blocks and granular form can also be applied in pre-prosthetic surgery to increase the volume of the alveolar ridge [49–51, 64].

In other studies concerning the biocompatibility of HAp, it has been shown they bind to bone and induces no toxicity or inflammation. HAp can bond with titanium implants, and the surrounding tissues to ensure a more rapid integration [64, 65]. Other researchers have applied nano-hydroxyapatite as a co-adjuvant material in oral surgery [49–51].

Recent studies suggest that nano-hydroxyapatite paste represents a promising class of grafting bone substitutes [50, 51, 66]. It has been suggested that nano-hydroxyapatite paste is a potent stimulator of cell activities, which probably

contributes to the fundamental process of periodontal tissue regeneration. Nanohydroxyapatite is an excellent source of free calcium and an important material finding growing applications in many other fields of dentistry [49, 64].

A growing interest in the material usage has prompted many researchers to look for new combinations that could improve existing materials or create new ones to foster regenerative applicability [49]. For example, the development of nanohydroxyapatite/polyamide66 complex have been investigated clinically and showed promising bone regenerative potentials associated with biocompatibility, osteoinduction and osteointegration [67, 68].

4.5 Application of Diatom-Based Nanocomposite for Bone Tissue Engineering

The diatom is a photosynthetic microalgae which is widely found in marine, freshwater, and sediments in rocks and wet soils [69, 70]. They are excellent source of biogenic silica. There are several thousand species of diatom with different morphological characteristics, and sizes ranging from 2 mm to 2 μm [71, 72]. Diatoms have optimal and natural hierarchical structure and make them ideal as functional materials for several bionanotechnological and microsystems fabrications [73]. Diatomaceous earth or diatomite is the collection of fossil remains of diatoms. They have well-defined microscopic pores and well-defined channels that perforate its entire skeleton. Within these microscopic pores are nanoscopic pores of about 40 nm in diameter, creating high surface area of about 200 m^2/g with characteristic high adsorption and low density [69, 74].

4.5.1 Purification of Biosilica from Diatom

Given the fact that diatom biosilica is sourced mainly from diatomaceous earth (otherwise referred to as Diatomite) and living diatoms, the separation of the biosilica from diatom obtained from one of the above-mentioned sources may differ slightly. The composition of biosilica is estimated to be around 70–90% of diatomite. Diatomite also contains other impurities that may include oxides of iron and aluminum [69]. According to a study by Wang et al., an acid treatment method was used [69]. Briefly, diatomite samples were first washed in de-ionized water, then dried and finally calcined at 600 °C for 3 h. This process was then followed by leaching with acid concentration of range 1 to 5 M HCL at different time points (24–120 h) and then purified [69, 75].

For living diatoms, established methods of purification of biosilica include oxidant cleaning, O_2 plasma etching and baking [69], acid treatment [76], hydrogen peroxide [77, 78], nitric acid [79], and sodium lauryl sulfate/ethylenediaminetetraacetic acid

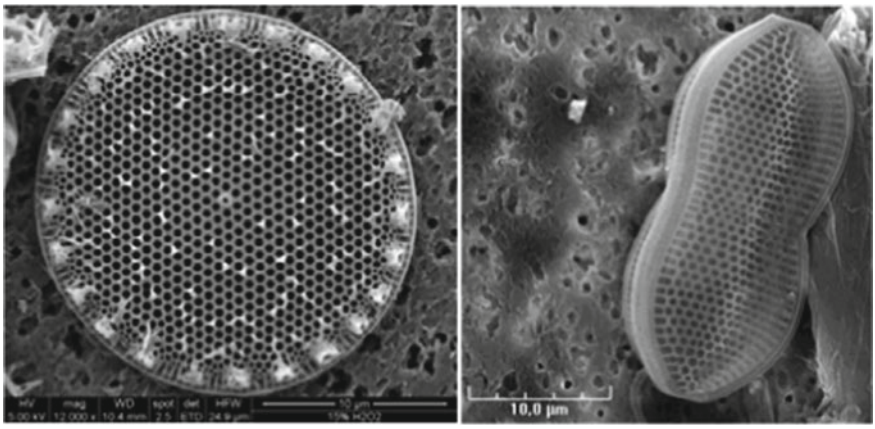

Fig. 4.4 Scanning electron microscopic view of the benthic diatom frustule showing pore distribution. Image modified and reproduced with permission from [81]

(EDTA) [80] have been reported. With each of these treatment methods, there is a possibility that the surface of the material may undergo structural changes. Consequently, the selection of method will be based on the targeted application. For instance, oxygen plasma etching may be the best method for cleaning frustule bound to the glass surface as showed in Fig. 4.4.

A rapid increase in diatom research over the past decade, has resulted in numerous publications in various of field of research specialties including development biology [82], protein synthesis [82], cell and molecular biology [83], development of biohybrid systems [84], biophotonics [85, 86], environmental science [87–89], optical biosensors [79, 90], cells, batteries and electroluminescent devices [91, 92] and tissue engineering [93].

Diatoms have intricate 3D assemblages of nanoscopic porous structures suitable for the development of artificial matrices and delivery of biomolecules [94]. The application of diatom biosilica is not widely researched; however, the regenerative potentials of diatom biogenic silica have been characterized for bone tissue engineering. For example, the diatom frustule, which is the main source of the biogenic silica, can be transformed into nanoparticles and applied for bone tissue engineering [95]. The potential regenerative indicators of diatom silica nanoparticles have been demonstrated by combination of silk fibroin to make a bone tissue engineering scaffold. The results indicated there is an increase in cell growth, and expression of key bone biomarkers associated with osteogenesis [93]. In addition, the biocompatibility assessment of diatom functionalized with different functional groups show no toxic effects, demonstrating the suitability of the material for the growing of cells in the development of artificial matrices for bone repair and drug deliveries [96]. A number of researchers have reported methods to culture diatoms in the laboratory [97, 98]. Further reading related to the biology and material properties of diatom can be found in [94, 99, 100].

4.6 Conclusion

Additional protocols may be necessary to transform the materials discussed in this chapter, including their nanostructured form, to fully utilize their regenerative potential for bone tissue engineering. These materials have promising regenerative properties applicable in various structural forms for bone repair or regeneration. The regenerative applications of diatom nanotechnology in bone tissue engineering is still emerging and more research into the regenerative mechanisms associated with the use of biogenic silica derived from diatoms needs to be further investigated.

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Chapter 5
Marine-Based Biomaterials for Tissue Engineering Applications

Innocent J. Macha, Besim Ben-Nissan and Wolfgang H. Müller

Abstract Marine organisms possess a vast range of properties, which portray a lot of their appropriate biomedical application potentials either directly, modified or as templates for biomimicking. It is and will remain a humble and smart move to learn from nature and try to copy faithfully the vital components so as to develop implantable biomaterials to mimic efficiently natural tissues or organs in order to substitute effectively diseased tissues or organs, to stimulate the body's own regenerative mechanisms, and eventually to promote tissue healing. The potentials of marine materials in tissue engineering and regenerative medicine applications are now evident. Recent advances in this area have shown to improve people's life.

Keywords Tissue engineering · Biomimicking · Coral · Drug delivery · Film composites

5.1 Introduction

The marine ecosystem is the largest ecosystem on the planet. It is estimated that 2.2 million different kind of species are present in marine environment although 91% of them still await description [1]. Marine species are classified into six kingdoms,

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bacteria, protozoans, chromista, fungi, plants and animals. Marine species are very uniquely produced from the exposure to exceptionally different oceanic environmental conditions. While it is true that a wide range of marine species are limited and protected, similarly there are also a variety of materials that are readily available and abundant and have yet to be exploited for their possible use. Marine organisms possess a vast range of properties, which portray a lot of their appropriate biomedical application potentials either directly, modified or as templates for biomimicking. It is and will remain a humble and smart move to learn from nature and try to copy faithfully the vital components so as to develop implantable biomaterials to mimic natural tissues or organs efficiently in order to substitute diseased tissues or organs effectively, to stimulate the body's own regenerative mechanisms, and eventually to promote tissue healing. Recently, there have been significant developments of marine-based biomaterials for various biomedical applications. Materials and natural design of marine organisms have been instrumental to introduce the simplest remedies to vital problems in regenerative medicine, providing frameworks and highly accessible sources of osteopromotive analogues, nanofibers, micro and macrospheres and mineralizing proteins [2]. Studies show that marine polymers such as polysaccharides have substantial biological properties that could be used as anti-inflammation, antimicrobial, anticancer and for osteoporosis treatment. Marine derived collagen has also been extensively studied for different applications in tissue engineering [3, 4].

In this chapter, the focus is directed towards the recent advancements of marine derived biomaterials and their applications in tissue engineering. Particularly, applications for hard and soft tissue are of interest.

5.2 Tissue Engineering Applications of Marine-Based Biomaterials

5.2.1 Hard Tissue Applications

More than 2 million procedures every year take place around the world for the treatment of bone fractures and defects from trauma or disease, making bone the second most commonly transplanted tissue after blood. Bone disorders and conditions incidences are currently increasing worldwide due to increasing of aging populations and poor physical activities. It has been reported that current clinical treatments for bone defects such as autologous and allogeneic transplantations using autografts and allografts have limitations and complications. It is a global priority to search for new bone regeneration techniques, which are perceived to be better than the current practice. The use of naturally occurring materials for this application will not only address the poor biological properties of most of the synthetic materials but also the cost pertaining from using them. Wide range of marine biomaterials has shown to be excellent candidates for tissue regeneration. The potential applications of natural biogenic materials such as marine structures can be easily overlooked due to the

environmental concerns. While it is true that a wide range of marine structures are limited and protected, similarly there are also a variety of materials that are abundantly available and are yet to be exploited for their possible use [5]. Previous work has shown that corals can be artificially grown as synthetic corals in specific areas and containers [6].

Collagen, richly present in marine species (Fig. 5.1), presents an interesting alternative source for pharmaceutical and biomedical applications. Extraction techniques of collagen from marine sponges such as Antarctic squid *Kondakovia longimana* and the sub-Antarctic squid *Illex argentinus* have been established with more than 50% efficiency [4]. Ferrario and his colleagues have shown that marine-derived collagen from different echinoderm (sea urchin, starfish and sea cucumber) has the potential to develop echinoderm-derived collagen membranes (EDCMs) for Guided Tissue Regeneration (GTR) which are thinner and mechanically more resistant than the commercial membranes [3].

Marine-derived scaffolds for bone tissue regeneration are regarded to be superior, because they are more biocompatible and provide more biointeractive sites for cell attachments and have structure that mimics the trabecular network of bone tissue. Mutsenko and his co-workers prepared chitin scaffolds derived from marine sponge *Ianthella basta* for tissue engineering applications [7]. They previously demonstrated that unique three-dimensional microporous chitinous scaffolds could be developed

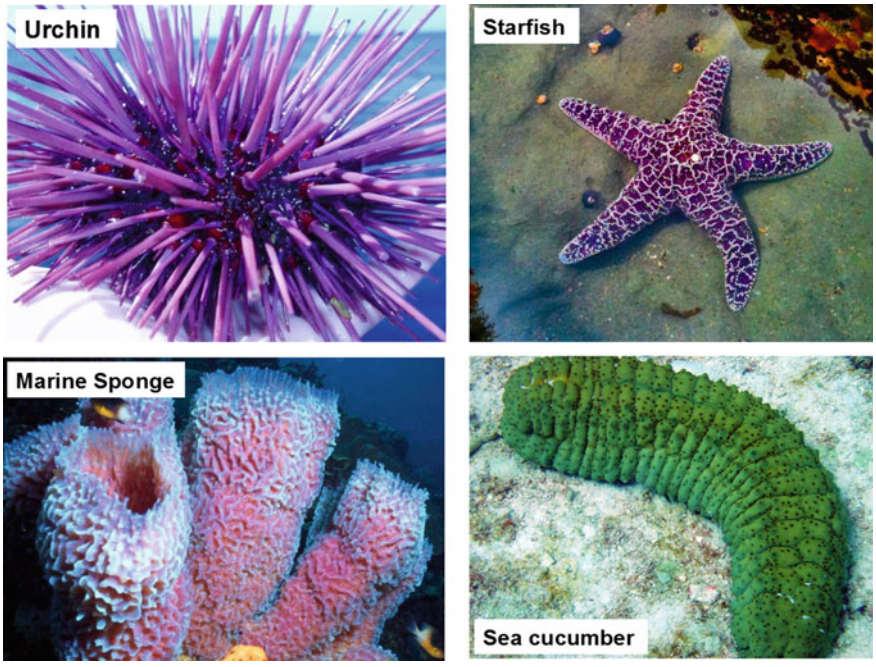

Fig. 5.1 Images of marine species richly in collagen

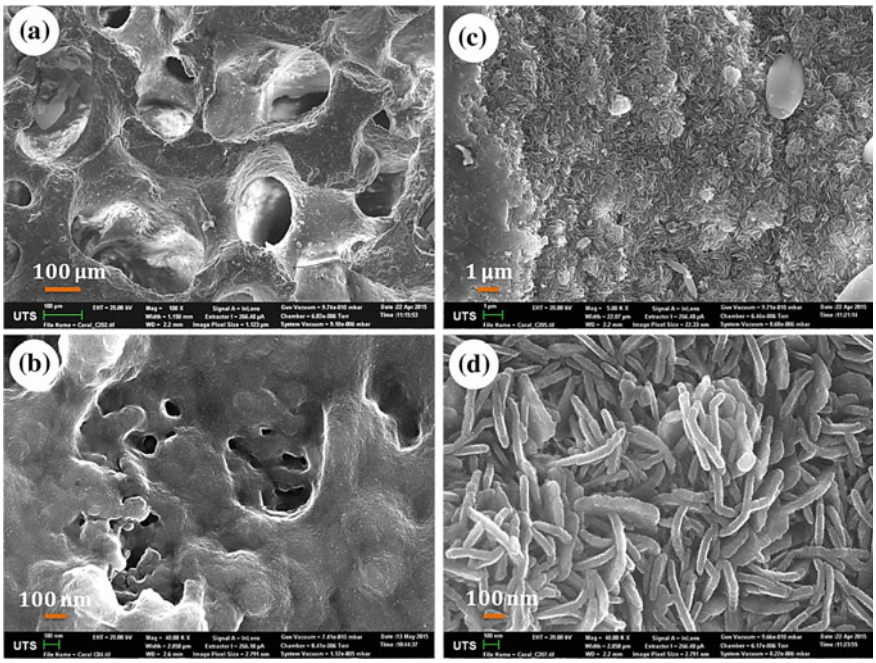

Fig. 5.2 **a** SEM picture of coral after conversion of **b** coral solid piece showing the retained porous morphology **c** higher magnification of coral solid piece before conversion for comparison, showing nano-pores, **d** higher magnification of converted coral piece showing platelets morphology of hydroxyapatite

from demosponge *Aplysina aerophoba* with excellent cytocompatible and biocompatible properties based on human mesenchymal stromal cells in vitro experiment [8]. In spite of having excellent biological properties, collagen scaffolds have poor mechanical properties that limit its applications. Attempts have been made to cross-link collagen scaffolds by chemical or physical methods or modified with natural/synthetic polymers or inorganic materials without compromising its biological properties [9]. A novel scaffold containing chitosan, hydroxyapatite has been derived from *Thunnus obesus* bone and marine sponge (*Ircinia fusca*) collagen with promising biomaterials properties for matrix-based bone repair and bone augmentation [10].

Other marine structures such as corals and shells have also potentials to be used as biomimetic scaffolds for bone tissue engineering and regenerative medicines. These structures have been converted to hydroxyapatite by different techniques [10–12] for a wide range of biomedical applications and will soon be used more extensively in bone grafting procedures. Coral-derived hydroxyapatite granules (HA) have been used in the treatment of periodontal bone defects, for filling congenital or surgically induced defects. There is repeated evidence of using marine structures with interconnected pores (Fig. 5.2) for bone defect treatment. Recently, open-cell scaffold from

sea urchin spines has shown a superior material properties for bone defect repair [13].

It was reported that new bone formed along the surfaces of scaffolds after implantation in rabbit femoral defects for one month and grows into the majority of inner open cell spaces. Post-operation after in three months showed tight interface between the scaffolds and regenerative bone tissue. Similar study by Zhan and his colleagues suggest that sea urchin spines converted to Mg-substituted tricalcium phosphate showed new bone formation around and inward the scaffold after implantation in rat femoral defects for 6 weeks [14].

5.2.2 Skin Tissue Application

Skin serves primarily as an essential barrier, protecting organisms from their environment [15, 16] and also plays a role in thermoregulation. Several strategies are available for the treatment of skin damage such as autografts, allografts, wound dressing and tissue engineering substitute. Particularly, deeper dermal wound of more than 4 cm diameter needs grafting for the treatment. It is globally estimated that burns caused 265,000 deaths every year. The need for a complete functional skin substitute is just increasing for skin repair and regeneration. Efforts have been focused towards developing skin substitute made up of artificial and natural materials. However, the skin substitutes currently available are not fully functional. New approaches for tissue engineering including injecting growth factors and extracellular matrix are being practiced towards tissue re-growth and wound healing [16]. A promising approach is suggested to use marine structures/species as a template for developing synthetic skin tissue [2]. The use of natural bionic materials will guarantee excellent biological effects from the biomimic extracellular cell matrix structure, hydrophilicity, and the multiple amino acids of marine derived compounds. The ideal synthetic skin substitutes should have close similarities to normal skin in terms of biological, physical and mechanical properties. At present, marine bioactive materials are extensively exploited as potential biological compounds for pharmaceutical and tissue engineered skin substitutes. Studies have shown that marine organism-derived collagen scaffolds are highly absorbent, elastic, and resistant to high temperature and bacterial attack thus high potential in clinical applications [17]. Biomimetic Tilapia collagen nanofibres stimulated the skin regeneration rapidly when implanted in sprague-dawley rat.

5.2.3 Cardiovascular Tissue Applications

Heart and blood vessel disease also called heart disease includes numerous problems, many of which are related to a process called atherosclerosis. Atherosclerosis is a condition that develops when a substance called plaque builds up in the walls

of the arteries. This build-up narrows the arteries, making it harder for blood to flow through. If a blood clot forms, it can stop the blood flow. Cardiovascular diseases include heart attack, heart failure, arrhythmia and heart valve problems. Efforts for discoveries of the treatments of cardiovascular diseases have been focused on analysing natural products. Marine organisms such as invertebrate's derived natural products are suggested to have pharmacological properties. Marine lipids, especially eicosapentaenoic acid EPA and docosahexaenoic acid DHA, have largely demonstrated their bioactivity in human health. Previous studies in the Inuit Eskimo during 1960s and 70s suggested that lower incidence of cardiovascular pathologies in that populations was associated with high intake of fish in their diet. Similar studies from other regions particularly in Iceland and Alaska on native populations showed similar relationships. It has also been shown that marine omega-3 polyunsaturated fatty acids (PUFA) have been shown to alleviate metabolic disorder symptoms, such as heart diseases, diabetes obesity and insulin resistance. It has been reported that Halichlorine from sponge *Halichondria okadai* is an inhibitor for the expression of vascular cell adhesion molecule and may thus impede atherogenesis [9, 14].

5.2.4 Liver Tissue Applications

Every year, millions of patients suffer from severe liver diseases and failure. Exogenous transplantation has become only a partial solution due to the limited number of organ donors. Advances in liver physiology, stem cell biology and tissue engineering have shown great promises in the development of cell-based therapies for treating liver disease and liver failure. Cell-based therapies have been proposed as an alternative to whole organ transplantation, as a temporary bridge to transplantation, and/or an adjunct to traditional therapies during liver regeneration. The three main approaches that have been proposed are: transplantation of isolated hepatocytes, implantable tissue-engineered constructs, and perfusion of blood through an extracorporeal bioartificial liver device containing parenchymal liver cells called hepatocytes. Despite significant investigations into each of these areas, progress has been stymied due to the propensity for isolated hepatocytes to rapidly lose viability and key liver-specific functions upon isolation from the native microenvironment of the liver [18]. Tissue-engineered scaffold from marine organisms could save the appropriate microenvironment for regeneration of fully functional hepatocytes from human embryonic stem cells [19]. There have been also other research focusing on various gelatin based composites scaffolds and microspheres such as chitosan/gelatin, silk fibroin/gelatin to enhance hepatic cell functions. By using 3D bioprinting microtechnology tools and biomaterials, it is now possible to synthesize 2D and 3D hepatic microenvironments to study hepatic cell functions and interrogate to model human diseases.

5.3 Drug Delivery Vehicles

Slow drug release has been an important research subject in the field of drug delivery for decades. Drug release systems have been proved to provide an outstanding alternative to conventional clinical therapies. With the advancement in both science and material design and engineering, more sophisticated therapeutic agent release systems have been developed with improved capabilities and performances for the treatment of resilient diseases such as musculoskeletal disorders and bone diseases. Drug delivery technology presents an interesting interdisciplinary challenge for pharmaceutical, chemical engineering, biomaterials and medical communities [20]. In general, a biomaterial that will act as a drug carrier must have the ability to incorporate a drug, to retain it in a specific site, and to deliver it progressively over time to the surrounding tissues.

In order to allow greater potency and less toxicity to healthy tissue, therapeutic agent release systems are required to control the release rate of the drugs locally, in order to maintain a desired drug concentration level without reaching a toxic level or dropping below a minimum effective level capability, which cannot be attained by conventional systemic administrations. The major challenges though are centred on how to regulate the drug releasing rate in order to keep its concentration within the therapeutic window, how to personalise the dosage regime for different people and the effective way to target affected tissues while keeping healthy ones spared. Using biodegradable materials in designing drug release devices addresses these challenges by providing the outstanding capability of performing localized and controlled delivery of drugs to different parts of the host body.

5.3.1 Marine Structures as Drug Carrier

Recently it has been demonstrated by Ben-Nissan and co-workers that marine shells with specific microspherical design offer desired functions for the delivery of Bisphosphonate (pamidronate) and antibiotic (Gentamicin) [21]. This has been possible by virtue of its unique structure and architecture of the foraminifera shells which are extraordinarily difficult to manufacture with the current know-how [22]. A combination of coralline derived hydroxyapatite loaded with clinically active substance and polylactic acid to form thin film composites has been suggested to improve drug stabilization and higher drug encapsulation efficiency of the composites [23, 24]. Coral derived bioceramic has clinical benefits due to its unique architecture and the presence of essential elements such as calcium, magnesium and strontium, which are important for the regeneration and repair of diseased bone tissue (Fig. 5.3).

Marine diatoms make up an important group: they contribute to approximately 40% of primary productivity in marine ecosystems and 20% of global carbon fixation [25]. Diatoms are bio-derived silica organism made up of amorphous silica in the process mediated by the enzyme silicatein through the formation of various

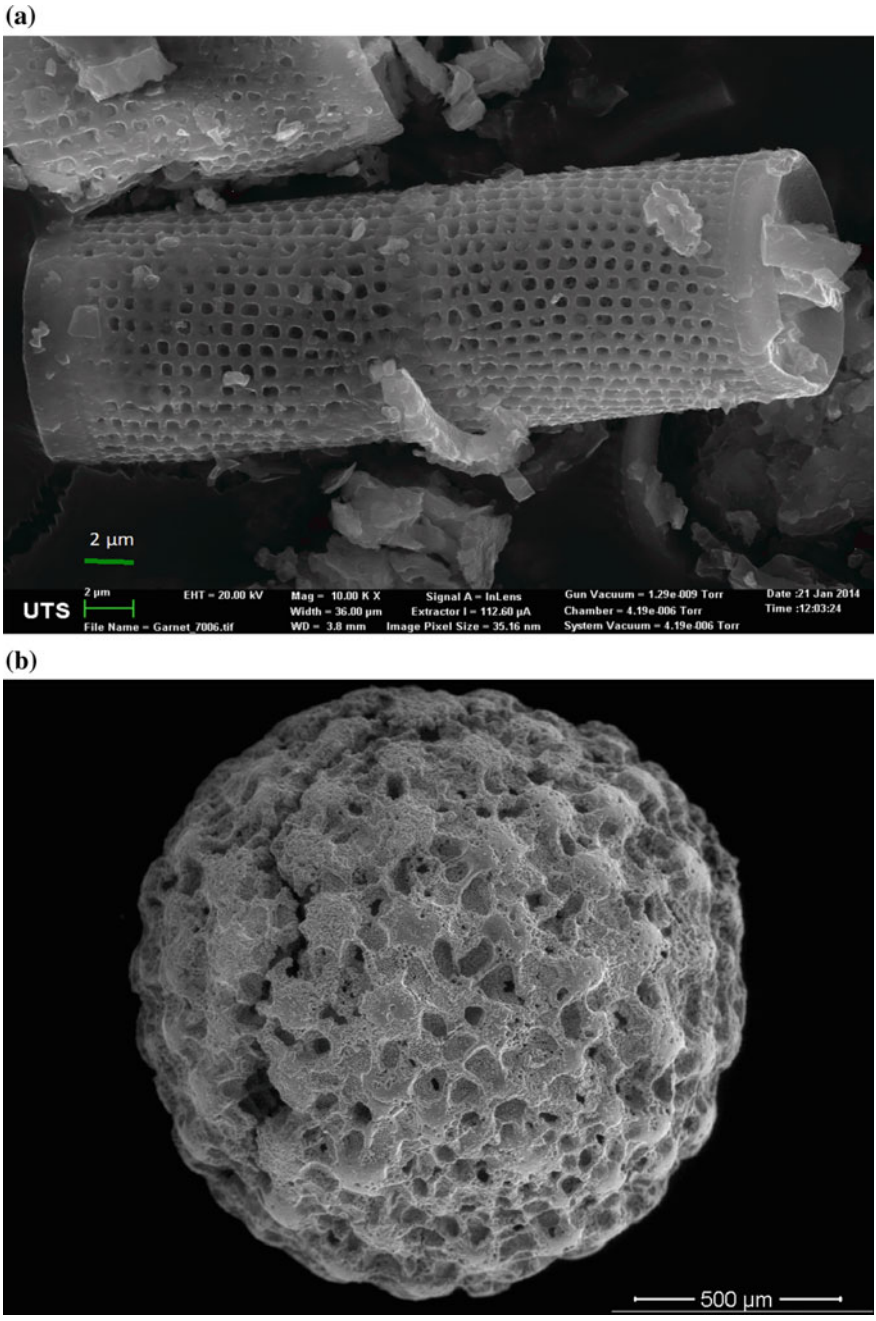

Fig. 5.3 SEM images of **a** diatom and **b** foraminifera marine structures

concentric layers. Among all these bio-silicifying organisms, sponges and diatoms are the two most important sources. Diatoms are of great interest for the development of new strategies in nanotechnology and molecular assembly as they provide modes of construction at these scales that could benefit the development of lab-on-chip biosensors, new generation drug delivery devices and bioreactors [2]. Diatoms are photosynthetic secondary endosymbionts found throughout marine and freshwater environments and are believed to be responsible for around one-fifth of the primary productivity on Earth. Diatoms are also used in nanotechnology to produce living nano-scale structures because they can build a silica shell at room temperature from a very small amount of silica dissolved in water. Due to its microscopic size and articulated internal pore structure, diatoms are suggested to be a good candidate for drug delivery. Biomimic of its structure to fabricate nanostructured templates has been reported for nanoimprint processes.

On the other hand, foraminifera because of their diversity, abundance, and complex morphology, fossil foraminifera assemblages are useful as bioindicator, and can accurately give relative dates to sedimentary rocks including coral reef health. Foraminifera have many uses in biomedical field and are used as precursors to calcium phosphates and drug delivery devices, because of their propensity to uptake and release clinical active substances due to the porous structure.

5.3.2 Scaffold for Drug Delivery

Biomimetic has provided us with the new directions in designing biomaterials with more functionality using less energy and materials. By using biogenic materials both organic and inorganic it is now possible to design complex tissue-engineered constructs for a wide range of applications. Marine skeletons have the potentials in the development of regenerative medicines in dentistry, orthopaedic and drug delivery systems. They present a richness of framework designs that act as landmarks and inspiration for further developments and the investment of the private biomedical industry to translate advanced functional biomaterials into clinical applications. It has been shown that self-sustaining musculoskeletal tissues can be supported by coral and marine sponge skeletons, and bone mineralization can be promoted by the extracts of spongin collagen and nacre seashell organic matrices [26]. Clinically active substances can be incorporated into nano and meso pores of marine origin skeletons and slowly delivered to a local or targeted area. Natural marine spheres loaded with drugs can spontaneously degrade and progressively release the entrapped biological contents relatively slow for an extended period of time [27, 28]. Tissue-engineered constructs that are generated by using template-mediated mineralization chemistry with morphology and patterning mimic plankton shells, provide many distinct advantages for tissue engineering as a physical template and a devices for controlled release of bone morphogenic protein (BMP), genes and growth factors.

5.3.3 Inflammatory Drug Delivery

Inflammation is one of the complex biological response occurs due to the detection of pathogens or tissue damage characterized by vasodilation, increased blood flow, vascular permeability and cellular extravasation [29]. Most of anti-inflammatory engineered drugs available today such as non-steroidal anti-inflammatory drugs (NSAIDs), glucocorticoids and immunomodulatory drugs have severe side effects range from gastric ulcers to kidney damage and death [30]. Marine invertebrates have potentials as secondary metabolites with pharmacological properties and can lead to the formulation of novel drugs. Indole alkanoids from marine invertebrates have reported to have anti-inflammatory effects with no severe side effects. Studies on conicamin from tunicate, lepadiformines A&B from ascidian and aplysinopsin-type compound from marine sponge showed anti-inflammatory activity [31, 32].

5.4 Conclusions

There is huge need for a complete functional tissue for treatment and repair of damaged tissues. Drug delivery systems and alternative drugs from natural resources present the possible and effective solution to the treatment of a wide range of diseases. The use of marine organisms and structures will guarantee excellent biological properties with similarities to normal hard or soft tissues in terms of physical and mechanical properties.

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During in his formative years Prof. Ben-Nissan worked on Titanium and its alloys and Magnesium alloy development and casting technologies and their properties for both engineering and medical applications. Over the last four decades, Professor Ben-Nissan has worked and contributed to the biomedical materials, implant design, production and analysis of various advanced ceramics, nano-coated sol-gel developed thin films, coated orthopedic and dental implants, anti-microbial slow drug delivery devices and methods, marine structures for clinical applications, biomechanics and finite element analysis of medical materials and engineering structures.

He has successfully developed materials for implant technology such as ceramic knee prosthesis, calcium phosphate based bioactive materials, bone graft production and bio-composites, and conducted research on biomechanics and modelling such as jaw bone, knee and hip joints, reliability and implant design modular zirconia ceramic knee prosthesis, femoral head and taper stresses and artificial ocular implants and bionic eye and recently on 3D printing of bioceramics and metallic implants and anti-microbial multifunctional coatings for drug delivery which are supported by the European Commission and the Australian Academy of Science research grants.

Since year 2000 he has published over 200 fully refereed papers in journals, and a book and 43 book chapters. He edited a book on Calcium phosphates and working on a second one on the use of Marine Structures in the Biomedical field. He is the editor of the Journal of the Australasian Ceramic Society.

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Chapter 6
Production and Characterization of Calcium Phosphates from Marine Structures: The Fundamentals Basics

Semra Unal, Oguzhan Gunduz, Sibel Akyol, Besim Ben-Nissan and Faik Nuzhet Oktar

Abstract Processes such as traditional wet chemical methods and heat and pressure-based hydrothermal methods are some of the important methods that has been used to produce hydroxyapatite (HAp), tricalcium phosphate (TCP) and other phosphate derivatives. Recently, new approaches such as ultrasonication (can be considered as a mechanical processing route) and hot-plating (heating element added to mechanical processing) were introduced. Using these new approaches, production for nanostructured calcium phosphate can be easily achieved. Traditionally, calcium phosphate-based compounds are produced using starting materials that contains calcium and phosphate. However, the use of marine structures as raw ingredients has been widely encouraged and their uses in the medical/surgical arena are creating new avenues such as in applications that support bone repair and regeneration. Primary sources of marine-based materials are numerous and includes corals, algae, cuttlefish, fish bones, sea urchin, sea snail shells, sponges, sea shells, foraminifera, barnacles, nacre, sea mussels and so on. Knowing other chapters in this book covers the review of these materials in more detail, therefore in this chapter, we aimed to give examples and review some of the new as well as traditional production routes and techniques used to characterize the physiochemical properties of the calcium phosphates produced are also examined.

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Keywords Hydroxyapatite · Calcium phosphates · Nanostructures · Marine sources · Bone · Sea shell · Characterization · Production routes

6.1 Introduction

6.1.1 Hydroxyapatite and Calcium Phosphates

It is estimated that the financial costs of repairing and replacing 280,000 hips, 700,000 vertebral and 250,000 wrist fractures using implants and/or biomaterials is approximately US$10 billion per year. This has led to a significant increase in demand within the surgical and biomaterials markets. Hydroxyapatite (HAp), one of the most popular and widely accepted biomaterials that is primarily used in maxillofacial and orthopedic surgeries, dentistry, and for other different purposes [1], has become widely accepted as a key scaffolding bone graft material. The biomaterials market is projected to reach $USD 149.17 billion by 2021. It has been reported that the market has already reached $USD 70.90 billion in 2016.

HAp, as a calcium phosphate, is accepted to be as a major component of bone and is commonly used as bone substitutes for hard tissue restorations due to its excellent properties. HAp is highly biocompatible and osteoconductive and it accelerates the formation of new bone tissues around the implant by supporting the growth of osteoblast cells [2]. Furthermore, the incorporation of substitutional elements to its structure such as magnesium, strontium or zinc also plays an important role in enhancing the mechanical properties and growth of new bone as it has an influence on the resorbability of HAp [3]. It has been also considered that the calcification of the bone itself will be manipulated and hence its fragility can be altered. In some cases, on the other hand, a material with denser and a more solid structure that displays greater mechanical properties is required and should be used for instances such as replacing long bones and in high load-bearing applications than utilizing the denser but less porous structured HAp.

Hydroxyapatite, either porous or dense, can be produced synthetically using chemical of the highest purity. In addition, HAp can also be manufactured using various animal-based materials such as bovine [4] and marine-based materials. Synthetic methods that are being used to create HAp includes precipitation [5], ultrasonic precipitation [6], microwave [7, 8], self-propagating combustion synthesis (SPCS) [9, 10], nano- and micro-emulsion systems [11, 12], the sol gel method [13–15], solid-state manufacturing technique [16], and hydro- and solvo-thermal methods [10, 17–19].

In addition to the synthetic production routes, conversion from marine structures have gained attentions over the past few years and the most popular marine-based materials used for HAp production are from various sea-shells [20], sea-snail shells

[6], sea-urchins [21, 22], barnacles [1], limpets [23], cuttlefish [2], corals [24] and fish bones [25]. Typically, sea shells possess aragonitic sub-structures, which can be convert to HAp and other calcium phosphate-based structures using certain production techniques.

The aim of this book chapter is to define parameters for the optimization of biomimetic HAp and calcium phosphate synthesis and processes. The marine resources included, which were proposed in this study are fish bones, fish scales, corals, cuttlefish bones, other shellfish, and finally from some marine algae. The proposed natural resources are very suitable candidates for preparing bone substitutes resembling the inorganic (mineral) component of natural bone tissue.

6.1.2 Hydroxyapatite Derived from Marine Sources

Majorities of HAp and calcium phosphate materials that are being used today are synthetically produced and there are a number of techniques have been applied to produce HAp, tricalcium phosphate (TCP), and biphasic calcium phosphate (BCP) materials in the form of powders, cements, thin films, or scaffolds. Similarly, a series of processes have been developed for obtaining HAp from natural sources. In the next sections, a detailed description will be given of HAp and/or calcium phosphate biomaterials, which are prepared by using marine sources as starting materials. Several sources have been identified and used for isolation of HAp such as fish bones [26], fish scales, corals, cuttlefish [2] and other seashells [6], and finally some marine algae.

Fish bone, if they can be artificially grown in a controlled environment to prevent heavy metals and other toxic material, may serve as an important source for biomedical applications owing to the presence of HAp as the major inorganic constituent (Fig. 6.1).

Considerable interest has been given to fish bone for the production of HAp due to the number of advantages associated with their use as a starting material. As a value, fish bones represent a significant part of the fish equaling to about 10–15% of total fish biomass (bones from the head up to the vertebrae). Therefore, the use of the fish bones for HAp production could bring huge benefits as high value-added products in many applications including tissue engineering [28, 29], biosensors [30, 31], and as drug carriers [32].

In contrast to fish bones, the main component of corals, cuttlefish bones, seashells and sea algae (*Rodophycophyta*) [33] consist not of fluoro-HAp or any other phosphate compounds but calcium carbonate ($CaCO_3$), usually in the form of aragonite. Consequently, the conversion of calcium carbonate structures to calcium phosphate is essential in the production of HAp, with the utilization of suitable phosphorus-based reagents. For instance, the sea algae (*Rodophycophyta*), a common seaweed, is characterized by a high content of $CaCO_3$ in their vegetative structure [33].

The most commonly used technique of converting aragonitic structures to HAp and other calcium phosphatic materials is hydrothermal transformation (HT). With

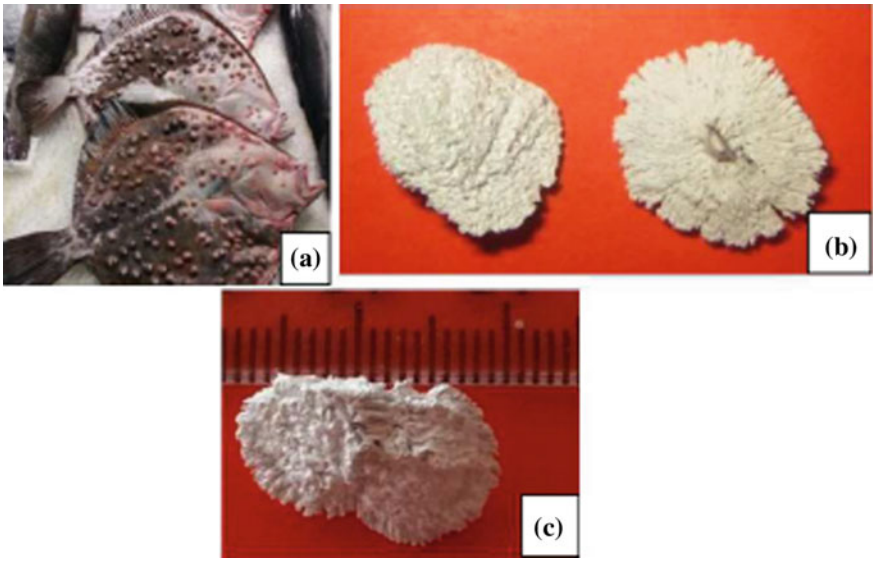

Fig. 6.1 Turbot (*Psetta maxima*). **a** Sold on the market bench; **b** calcined bulky bumps; and **c** bumps with millimeter scale (reprint with permission from [27])

this approach, the aragonite components of the sea creatures, such as the bone of cuttlefish or nacre materials (*Mytilus galloprovincialis* and *Ostrea edulis*) are transformed [2, 34]. Prior to any hydrothermal transformation, the aragonite ($CaCO_3$) content of the sea structure such as cuttlefish [2] or nacre [34] must first be calculated using differential thermal analysis method (DTA/TGA). Without this calculation, it is impossible to estimate the amount of chemicals needed for the transformation. The required amount of calcium phosphate solution was calculated using the equation stated below [2, 34], which was first postulated by Roy and Linhagan [35]:

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

Hydrothermal treatment, in which the aragonite conversion was carried out, involves the use of a stand-alone high pressure and temperature vessel or it can be carried out in a high temperature-resistant metallic crucible with an appropriate seal and placed inside an oven at high temperature. In some vessels and systems, a polytetrafluoroethylene (Teflon) liner is used to prevent ion release from the crucibles.

The furnace is heated a temperature between 1000 and 1400 °C. This method is carried out under an unpredictable amount of pressure. The process may seem slightly dangerous, in particular, the steal container could explode due to the difficulty in controlling the amount of internal pressure inside the vessel during the reaction. On the other hand, the right amount of pressure suitable for HAp formation can be

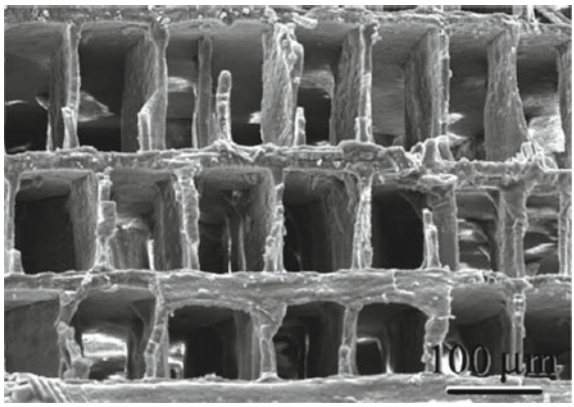

Fig. 6.2 Typical porous microstructure of treated (1250 °C) HAp-scaffolds [2]

achieved with the correct quantity of solutions and suitable temperatures (Fig. 6.2). Nevertheless, Parr reactor or similar reactors have pressure control devices and valves to vent any unwanted high pressures.

In addition to hydrothermal methods, a number of aragonitic structures such as cuttlefish bone (*Sepia officinalis*) [2], corals, sea snail shells [36], sea urchin shells [21], several mussel shells [20], sweet water pearl powder [37], sea algae [33] has been successfully transformed into various calcium phosphate bioceramic powders using mechanochemical methods.

Mechanochemical method consist of an ultrasonic cleaner and a simple hot-plating stirring equipment. Hot-plate stirring equipment is used to achieve uniform mixing of powders within the aqueous solution. Although mixer design begins with a focus on process requirements, the mechanical design is essential for successful industrial operation. A magnetic hot-plate stirrer is an equipment that has been used to create appropriate mixing [38].

The primary mission of the ultrasonic cleaner is to provide a cleaning service but vibrations within the equipment create vibrating-mixing movements. In the literature, this process is referred to as hot-plating and/or ultrasonication. Those two terms can also be used to describe the mechanochemical conversion. Sahin et al. had performed ultrasonication on Tiger Cowrie (*Cypraea tigris*) and were able to obtain nanostructured HAp and tricalcium phosphate bioceramics [6]. Furthermore, a study by Ağaoğullari et al. utilized both ultrasonic and hotplate methods to obtain very delicate monetite and tricalcium phosphate from sea urchins [21].

6.2 Synthesis and Preparation Methods

6.2.1 Hotplate Method

This relatively new method is also practical and is less expensive compared to other production method. The hotplate method is comprised of several stages:

(1) The solutions were transferred on a hotplate-mixer at 80 °C for 15 min.
(2) Next, an equivalent amount of concentrated H_3PO_4 to the amount of $CaCO_3$ determined within each seashell species were added dropwise to the prepared solutions.
(3) **For the production of tricalcium phosphate**: the quantity of H_3PO_4 is calculated to achieve a stoichiometric molar ratio of Ca/P equals to 1.5. This is to ensure a quantitative condition for the transformation of $CaCO_3$ to tricalcium phosphate is created.
(4) **For the production of HAp**: the amount of H_3PO_4 is calculated to achieve a stoichiometric molar ratio of Ca/P equals to 1.667. Again, this is to ensure a quantitative condition for the transformation of $CaCO_3$ to HAp is created.
(5) The reactions (for both the production of tricalcium phosphate and HAp) were left to evolve for 2 h. Afterwards, the mixtures were dried at 100 °C for 24 h to evaporate the liquid chemical reactants [39].

6.2.2 Ultrasound Method

Similar to method described above but this technique utilized a different agitation and mixing approach. Suspensions of raw powder from each sample were placed in an ultrasonic bath and the temperature was set to 80 °C for 15 min. Subsequently, the method follows the same procedure as stated for the hotplate method including the addition of appropriate volumes of H_3PO_4 solutions [39]. Furthermore, calcination was performed at two different temperatures. The raw HAp was calcinated at 800–850 °C, while the raw tricalcium phosphate was calcinated at 400–450 °C.

6.2.3 Thermal Calcination Method

Using the thermal calcination method, naturally occurring hydroxyapatite-based materials such as fish or crocodile bones can be converted. The following method describes the conversion process for raw fish bones.

Raw fish bones were boiled in clean water to remove any traces of flesh and skin. The boiled bones were then washed and mixed with 1% sodium hydroxide (NaOH) and later with acetone to remove proteins, lipids, oils, and other organic impurities.

After thorough washing, the bones were grounded using a mortar and pestle and then dried for 24 h at 60 °C. In the thermal calcination process, bone was placed in a silica crucible and heated to a temperature of 900 °C in an electrical muffle furnace under air atmosphere for 5 h [40].

In another study, bones of Brazilian river fish such as pentado (*Pseudoplatystoma corruscans*), jaú (*Paulicea lutkeni*), and cachara (*Pseudoplatystoma fasciatum*) were initially calcined at 900 °C for 4–12 h followed by crushing to a powdered form using high-energy ball mill at two different milling times (2 and 4 h). SEM analysis indicated that the milling time affected the size of produced spherical particles. Elemental analysis confirmed the presence of Fe^{2+}, Cr^{3+}, Ni^{2+}, Mn^{2+}, Cu^{2+}, Zn^{2+}, K^{+}, and Na^{+} as trace elements; however, the presence of first four ions was attributed to the use of stainless-steel milling balls [41]. In later study by Coelho et al. [42], natural HAp was processed by calcining the bones at 900 °C for 8 h. Calcination for 8 h was determined to be the most suitable at obtaining nanoparticles. They were hand crushed inside an agate crucible prior to undergoing milling in a high-energy miller [41].

A relatively cheap and easy approach was described by Boutinguiza et al. [43] capable of obtaining a significant quantity of HAp from fish bones. Dried fish bones from different species of sword fish (*Xiphias gladius*) and tuna (*Thunnus thynnus*) were calcined in a furnace at two different temperatures of 600 and 950 °C for 12 h. The calcined samples were milled for 1 min using milling balls. TEM analysis confirmed the isolation of rod-like particles with submicron particle size from both fish species. FTIR, Raman, and XRD studies confirmed the formation of HAp after calcination at 600 °C. In addition, the formation of β-tricalcium phosphate was confirmed using XRD and Raman spectroscopy after calcination at 950 °C. Deviation of lattice parameters from the standard HAp was hypothesized and attributed to the carbonate substitution [43].

6.2.4 Microwave Method

This method was utilized to convert calcium carbonate from seashells to calcium oxide utilizing a microwave source and heating to a temperature above 700 °C for an extended period. Basically, raw seashells were first cleaned using boiling water to remove all organic matter as well as other foreign materials and contaminants. After drying overnight in an oven at 80 °C, shells were weighed and then heated in a furnace at either 900 °C for 30 min [8] or at 1100 °C for 5 h [7]. The produced calcium oxide from the shells were finely-grounded into a powder. One gram of the produced calcium oxide was mixed with 0.1 M ethylene diamine tetra-acetic acid (EDTA) to create 0.1 M solution of Ca-EDTA complex. Next, 0.06 M Na_2HPO_4 was added dropwise to obtain Ca-EDTA complex and the solution mixture was stirred for 15–30 min. The pH value of the solution mixture was kept at 13 through the addition of NaOH solution. The mixture was treated with microwave (2.45 GHz, 1100 W) until the mixture was dried. The resultant precipitate was washed several

times using deionized water to remove possible Na and EDTA residues, and then it is dried in a vacuum oven at 80 °C for 6 h or at 110 °C for 5 h, to obtain HAp powders. Shavandi et al. successfully produced HAp powders from mussel shells using microwave irradiation method and obtained rod-like nano-crystalline HAp particles of 30–70 nm in length [8].

6.2.5 Hydrothermal Method

This method was applied on seashells, corals, mussels, algae, and all other calcium carbonate-containing raw marine materials. The method also can be used on non-marine-based calcium carbonate materials such as bones for a full or partial conversion to HAp for biomedical applications.

The grounded shells or other material were mixed with 2 M sodium hydroxide (NaOH) at 250 °C for 5 h (sodium hydroxide solution with solid/liquid weight ratio 1:30). 2 M sodium hydroxide (NaOH) was prepared with water as solvent. This procedure was repeated several times to ensure proper removal of organic moieties. The mixture was then filtered in a suction pump with continuous washing with water until the pH was neutral. The resultant product was dried in an oven at 100 °C [40].

In another study, raw shells were washed thoroughly in distilled water to remove salts and dirty substances. After the drying of samples in air, they were deproteinized through external washing with 0.1 M HCl and washed several times with distilled water. The remaining proteins or organic matter were treated with 5% (w/v) NaOH, heated at 70 °C for 5 h. The obtained fine white precipitate was washed with distilled water and further dried at 60 °C. The next stage of alkaline heat treatment involves the addition of 50% (w/v) NaOH to the treated powder and heated to a temperature of 100 °C and stirred for 1 h. After hydrothermal treatment, the obtained HAp nanopowder was washed thoroughly with deionized water until the washing solution became neutral and then dried at 60 °C [44].

6.2.5.1 Coralline Apatite by Hydrothermal Conversion

Hydrothermal conversion of coral to apatite is widely explained in other chapters in this book and will only briefly explained in here.

The coral was obtained from the Australian Great Barrier Reef. The coral was shaped in the form of a block, powders or microspheres and was treated with boiling water and 5% NaClO solution to remove any organic matter. Hydrothermal conversion was carried out in a Parr reactor at 220 °C and 3.8 MPa pressure with excess ammonium biphosphate. The resultant product is then cleaned and dried at 700 °C if the intended application is to be used as a bone graft [45]. Similar conversions were carried out on foraminifera microspheres for drug delivery applications.

6.2.6 Alkali Treatment Method

Synthesis of phase-pure nanocrystalline carbonated-HAp from fish (*Tilapia nilotica*) scale waste through alkaline heat treatment method was recently described by Kongsri et al. [44]. Thoroughly washed and dried fish scales were deproteinized and heated with 50% NaOH for 1 h at 100 °C to obtain HAp. FTIR analysis confirmed the replacement of some of the phosphate groups with the carbonate group (B-type substitution). ICP-OES confirmed that the Ca/P ratio was 1.67, same as the theoretical value [44].

6.3 Characterization Methods

6.3.1 Thermogravimetric Analysis

Prior to determining the amount of solutions required for conversions, the untreated raw nacre or coral materials must be treated by thermal analysis at temperatures ranging from 25 to ~900 °C prior to converting calcium carbonate-containing marine materials such as verting nacreous materials (*A. Mytilus galloprovincialis* and *Ostrea edulis*), designated for simplicity purposes hereafter as shells.

Most shells and coralline materials can undergo thermal analysis. After TGA/DTA analysis, the weight loss (TG curve) as a result of organic matter burnt off was approximately 2–3%. It is a well-known fact that the shells predominantly consisted of $CaCO_3$, whose decomposition begins at ~500 °C and finishes at ~700 °C, accounting for a weight loss of about 44% predicted using the following equation:

$$CaCO_3 \rightarrow CaO + CO_2$$

These results are in a fairly good agreement with other earlier studies. Two types (1 and 2) of organic matter has been identified through thermal analysis of nacres, corals and sea shells. The results of the Lemos et al. showed that the conversion produces nano-structured particles as shown in Fig. 6.3 [34].

6.3.2 Microscopy and Morphology

The morpho-compositional characteristics of marble, seashell and fish bone and the bioceramic products derived from those raw materials were identified by many researchers through SEM analysis coupled with EDS (EDAX). Results of analyses concerning the starting constituents, intermediate synthesized products, and the final bioceramic material derived from every category of resources found naturally have been reported in a number of articles and books related to marine technologies.

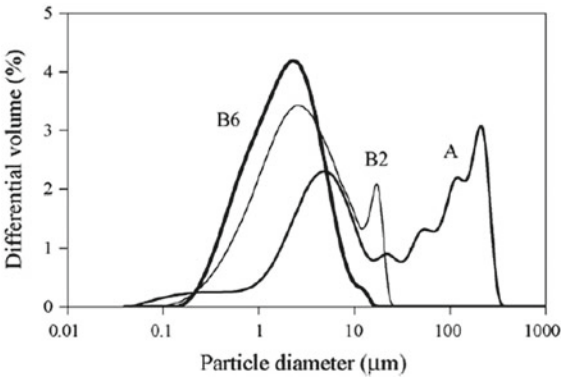

Fig. 6.3 Particle size distributions of the powders from two different shells designated as A and B shells. After milling for 2 h (B2) and for 6 h (B6) (modified from [34])

For example, SEM images of as prepared Sputnik sea urchin samples (Fig. 6.4a–d) and Trochidae samples (Fig. 6.5a–d) revealed flat-plate morphology identified as monetite and needle-like shape particles of HAp [39].

In the samples that were heat-treated to 850 °C, SEM images revealed porous whitlockite (β-MgTCP) plates at higher magnifications (Figs. 6.6 and 6.7); it can be estimated that the interconnected pores have dimensions of several hundred nanometers [39]. However, the dimensions of the calcium phosphate particles as revealed in the SEM images are within several hundred micrometers. Thus, these dimensions make such materials ideal for their intended applications in the biomaterials arena.

Microstructural features of fractured surfaces were investigated and, consequently, interesting trends were observed due to their morphology. It has been well established that the organic phases between the inorganic scaffolding is of vital significance. The typical microstructure at a fracture surface of the shells of a local sea snail (*Cerithium vulgatum*) is revealed at low magnifications in the SEM image as shown in Fig. 6.8.

Plate-like structure of the scaffold can be attributed largely to aragonite crystals. As shown in the SEM image (Fig. 6.9), the dimensions of the powders are approximately 200 nm. Some elongated rod-like prismatic particles with a length of 1–1.5 μm and a width of ca 200 nm are also observed [36].

A study by Ağaoğullari et al. revealed particles synthesized from sea urchin shell produces cube-like structures with dimensions of ca. 3 μm [21]. Furthermore, a comparative study by Venkatesan et al. has suggested that nanostructured particles can be easily obtained from *Thunnus obesus* during the isolation of HAp using both thermal calcination and alkaline hydrolysis method [40]. The study reported that nanostructured HAp crystals with a length of 17–71 nm and a width of 5–10 nm were observed after heat treatment at 250 °C for 5 h using the alkaline hydrolysis method. On the other hand, thermal calcination method provided particles with good crystallinity with dimensions of 0.3–1.0 μm once heat-treated at 900 °C for 5 h [40].

Oktar et al. [1] successfully produced natural tricalcium phosphate and HAp powders from barnacle shell (*Megabalanus tintinnabulum*) using mechano-chemical conversion method. The hot-plate approach produces HAp materials which are elongated

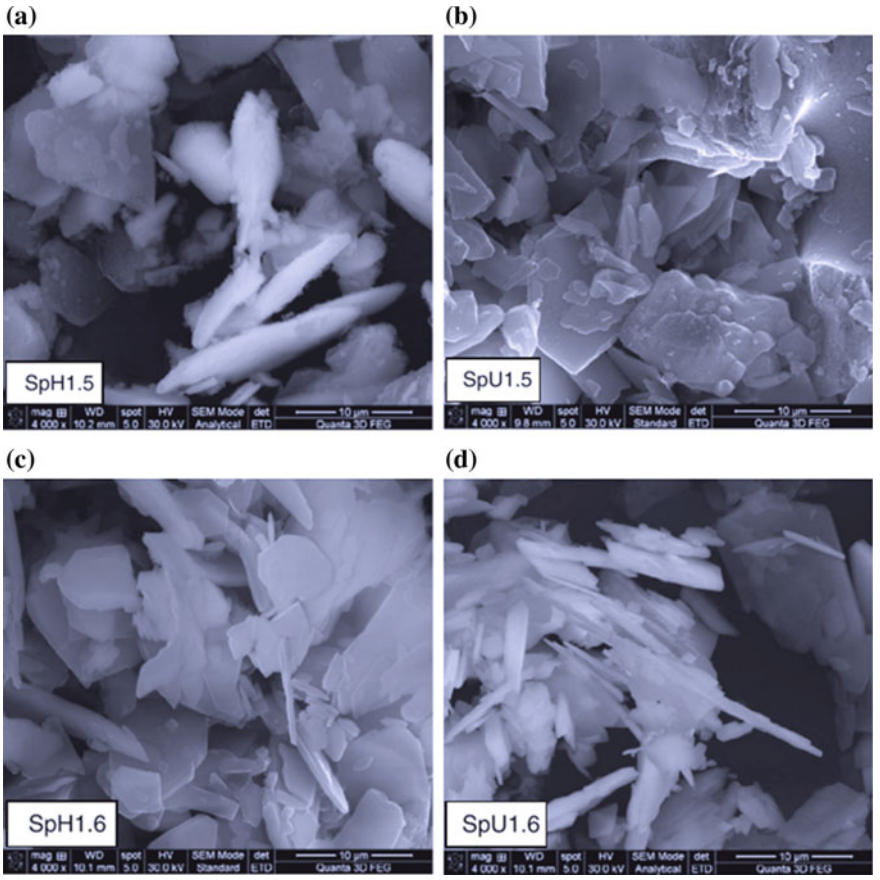

Fig. 6.4 SEM images of as-prepared Sputnik sea urchin (*Phyllacanthus imperialis*) samples (scale bar = 10 μm). **a** and **b**: at 1.5 Ca/P ratio (tri-calcium phosphate); **c** and **d**: at 1.66 Ca/P ratio (HAp) (reprint with permission from [39])

rods covered in conglomerates of 100–250 nm, while the combination of hot-plate and heat treatment created HAp that are spherical nano-clusters of approximately 200–300 nm in diameter. The results clearly demonstrate that the heating procedure changes the shape and morphology of the HAp crystals.

The EDS spectra of raw fish bone such as the European sea bass and *Dicentrarchus labrax* showed in addition to magnesium, there are other major elements such as Ca, P, C and O. In addition, the use of a naturally derived HAp with magnesium is more advantageous than synthetic analogs, which do not contain traces of magnesium. For this reason, the incorporation of magnesium permits cell proliferation, which is essential for the healing of bone fractures [3].

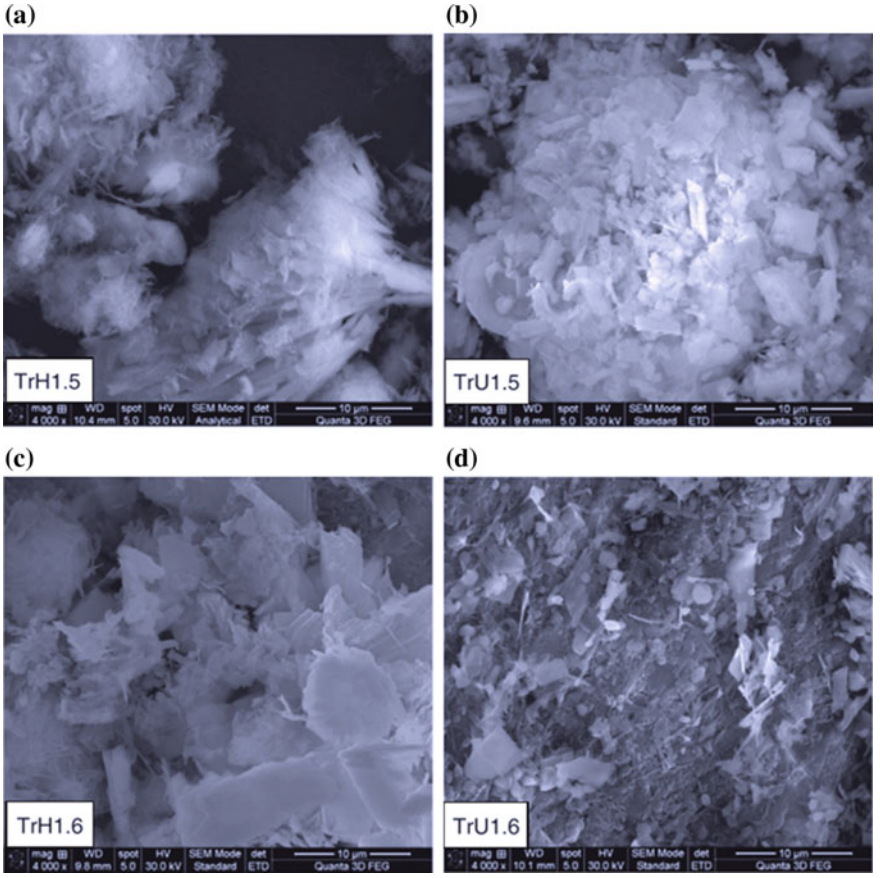

Fig. 6.5 SEM images of as-prepared *Trochidae Infundibulum concavus* samples (scale bar = 10 μm). **a** and **b**: at 1.5 Ca/P ratio (tri-calcium phosphate); **c** and **d**: at 1.66 Ca/P ratio (HAp) (reprint with permission from [39])

6.3.3 XRD and Phase Determination

To date, amorphous calcium phosphate, brushite, monetite, octacalcium phosphate, tricalcium phosphate, calcium pyrophosphate and apatite are the biologically relevant calcium phosphates that are well known. Furthermore, monetite ($CaHPO_4$), which is a mildly acidic calcium phosphate, has been used as a precursor during the synthesis of HAp [46] (Table 6.1). A study by Oktar et al. also produced the same crystallites using purple barnacle (*Megabalanus tintinnabulum*) [1] (Fig. 6.10).

In the giant purple barnacle (*Megabalanus tintinnabulum*), the monetite formation is clearly shown in Fig. 6.11. They are often considered as a precursor for tricalcium phosphate. It was also reported that monetite is a significant powder component in self-hardening calcium phosphate pastes which has been previously reported in a

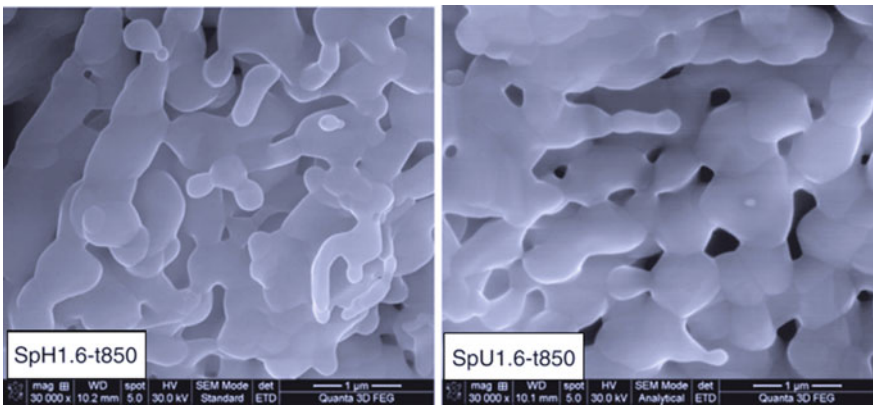

Fig. 6.6 SEM images at high magnification of Sputnik sea urchin (*Phyllacanthus imperialis*) samples heat-treated to 850 °C (scale bar = 1 μm) (reprint with permission from [39])

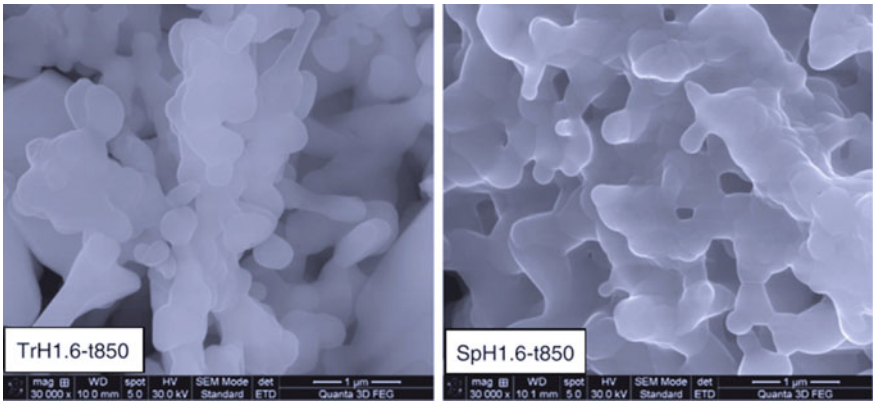

Fig. 6.7 SEM images at high magnification of *Trochidae Infundibulum concavus* samples heat-treated to 850 °C (scale bar = 1 μm) (reprint with permission from [39])

number of studies and could be used for skeletal repair [1]. A study by Macha et al. [20] also revealed the production of monetite from Mediterranean Mussel (*Mytilus galloprovincialis*) shells. In addition to monetite phases, other phases such as HAp, whitlockite, brushite, and calcite can be easily identified if the giant purple barnacle was examined very carefully [1]. Additional peaks in Fig. 6.11 revealed some of the other mixed $CaCO_3$ phases has retained after the conversion process [20].

Portlandite is a rare oxide mineral with the naturally occurring form of calcium hydroxide ($Ca(OH)_2$). Result from a study by Gunduz et al. has detected its presence in Atlantic Deer Cowrie shells (*Cypraea cervus Linnaeus*) [47]. It is the calcium analogue of brushite. It was used decades ago as biomedical ceramics due to its similarity to the mineral phase of bone. There is also another mineral Hilgenstockite,

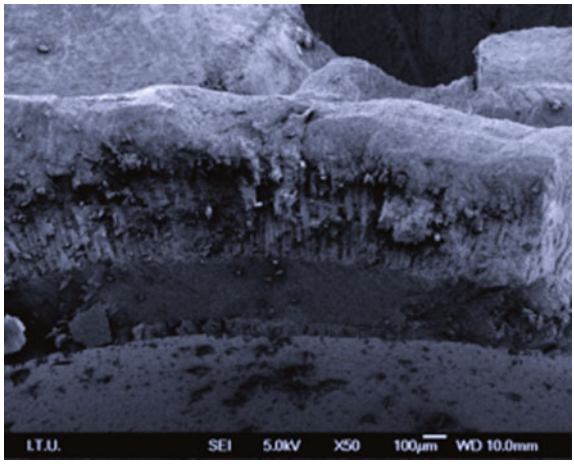

Fig. 6.8 Microstructure at fracture surface of *Cerithium vulgatum* shell (reprint with permission from [36])

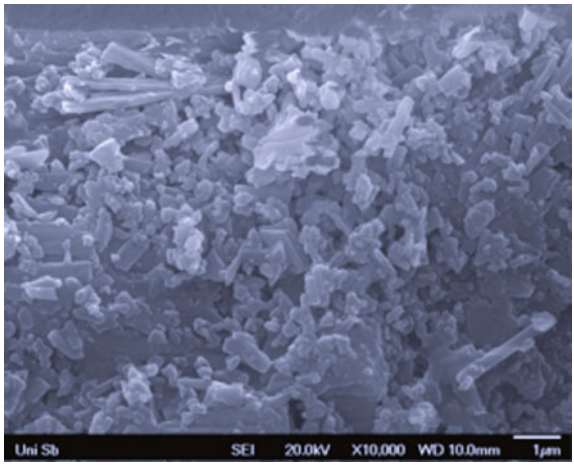

Fig. 6.9 SEM image of raw powder after planetary milling and sieving using a sieve of 100 µm (reprint with permission from [36])

which is a lesser-known form of calcium phosphate. Hilgenstockite is a tetracalcium phosphate with a chemical formula of $Ca_4(PO_4)_2O$ [47].

Whitlockite is discovered in *Nassarius hinia reticulatus* and it is a recognized tricalcium phosphate phase, as shown in Table 6.1. Tricalcium phosphate is a bioresorbable material and is more soluble than HAp [48]. In Fig. 6.12, various calcium phosphate phases were obtained during the calcination of Giant Purple Barnacle (*Megabalanus tintinnabulum*) including HAp, whitlockite, monetite, brushite and calcite [1]. Even though HAp is the major phase of hard tissue, a short-range ordered whitlockite phase ($Ca_{18}Mg_2(HPO_4)_2(PO_4)_{12}$) in an amorphous form also exists in human bone and dentin with an estimated amount of approximately 26–58 wt%, respectively, based on the amount of Mg.

Table 6.1 Different calcium phosphate phases and relevant Ca/P ratios [20]

	Mineral	Empirical formulas	Ca/P
Dicalcium phosphate dehydrate	Brushite	$CaHPO_4{\cdot}2H_2O$	1.00
Dicalcium phosphate	Monetite	$CaHPO_4$	1.00
Octacalcium phosphate		$Ca_8H_2(PO_4)_6{\cdot}5H_2O$	1.33
β-tricalcium phosphate	Whitlockite	β-$Ca_3(PO_4)_2$	1.50
Hydroxyapatite		$Ca_{10}(PO_4)_6(OH_2)$	1.67
Tetracalcium phosphate nonoxyde		$Ca_4(PO_4)_2O$	2.0
Defect apatites		$Ca_{10-x}(HPO_4)_x(PO_4)_{6-x}(OH)_{2-x}$ $0 < x < 2$	$(10-x)$:6

Fig. 6.10 Image of giant purple barnacle (*Megabalanus tintinnabulum*) (reprint with permission from [1])

6.3.4 FT-IR Analysis

FT-IR is an excellent physicochemical analysis tool ideal in obtaining a thorough understanding into the vibrational origin of the phosphate, carbonate and amide groups. There are a number of excellent review articles and chapters on the physicochemical analyses of calcium phosphates [49].

FT-IR spectra of converted marine structures showed the presence of phosphate (PO_4^{3-}), hydroxyl (OH^-) and carbonate (CO_3^{2-}) ions. Oktar et al. [1], Gunduz et al. [36] and Sahin et al. [6] prepared tricalcium phosphate and HAp from seashells for bone graft applications using the hot plate method, which showed the absorption bands at about 544 and 602 cm^{-1} corresponded to the PO_4^3 $\nu 2$ bending modes, the bands at ~960 cm^{-1} exhibited PO_4^3 $\nu 2$ bending modes and the band at ~1100 cm^{-1} indicated the characteristic reflection of the PO_4^3 $\nu 3$ vibrations. Moreover, the characteristic vibration bands of CO_3^{2-} group forms weak peaks at ~875 cm^{-1} ($\nu 2$ asymmetric bending), ~1400 cm^{-1} ($\nu 3$ asymmetric bending). As the process temperature

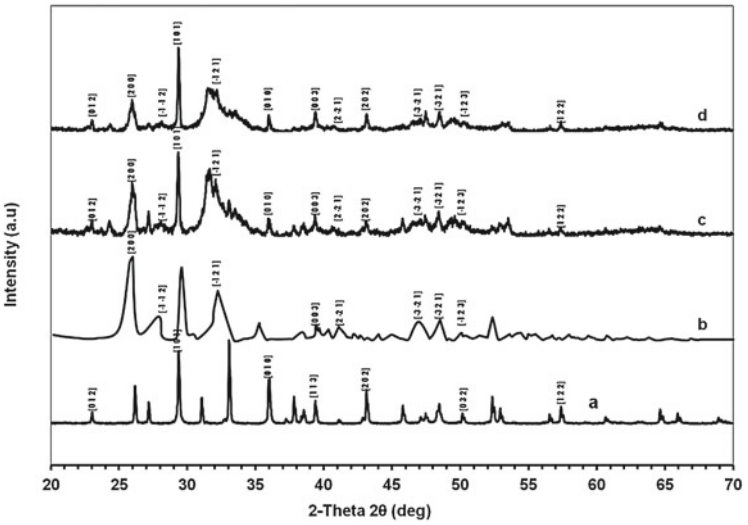

Fig. 6.11 XRD patterns of **a** mussel shell; **b** monetite; **c** monetite formed by HP (hot-plate) method; and **d** monetite formed by HPUS (hot-plate ultrasonic) method (reprint with permission from [20])

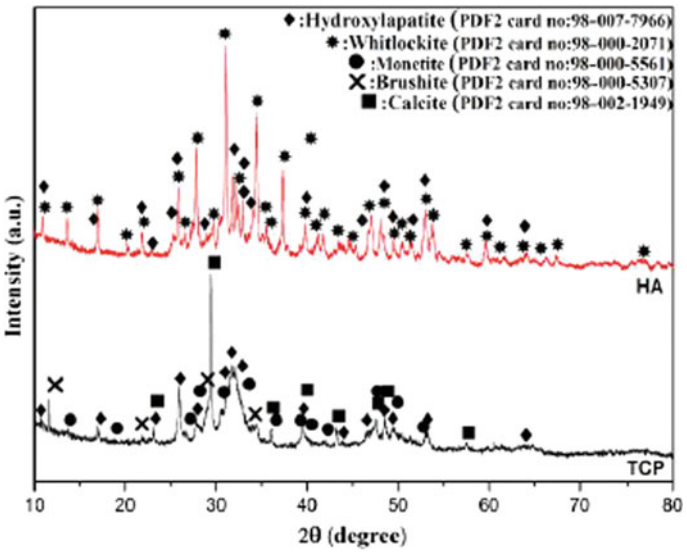

Fig. 6.12 X-ray diffraction analysis for hot-plated HAp material (heat-treated at 800 °C, upper trace) and X-ray diffraction analysis for hot-plated tricalcium phosphate material (heat-treated at 400 °C, lower trace) (reprint with permission from [1])

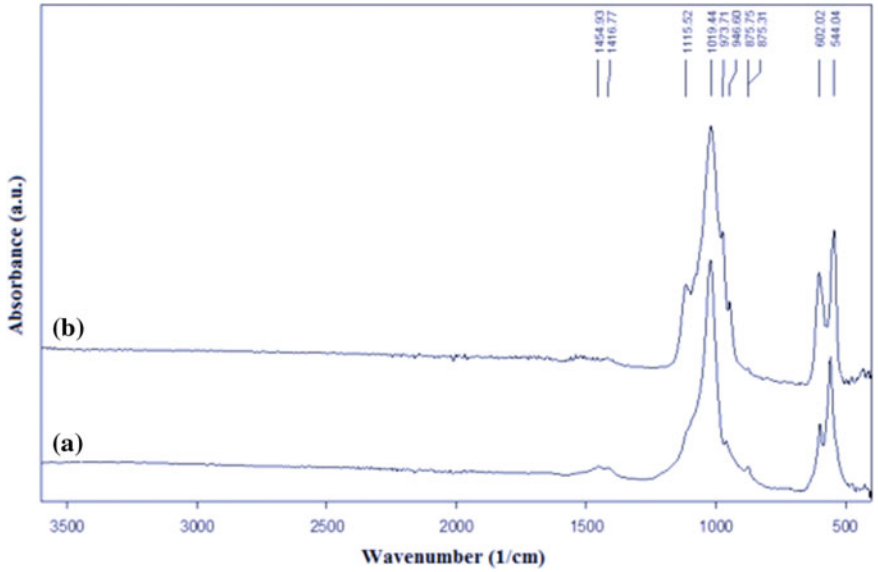

Fig. 6.13 **a** FTIR analysis for hot-plated tricalcium phosphate material (heat-treated at 400 °C); **b** FTIR analysis for hot-plated HAp material (heat-treated at 800 °C) (reprint with permission from [1])

increases, the intensities of the absorption bands for the PO_4^{3-} group become more distinctive and their characteristic peaks emerge more strongly (Fig. 6.13) [1].

In addition, Fig. 6.14a, b showed that an increase of the calcination temperature from 400 to 800 °C resulted in the appearance of peaks at 1500 and 3700-3500 cm^{-1}. The additional FTIR bands in the range of 3700–3500 cm^{-1} as shown in Fig. 6.14b were also observed in a study by Duta et al. [50], which was assigned as the O–H stretching vibrations of surface P–OH groups.

On the other hand, Boutinguiza et al. [43] reported that the vibration bands of CO_3^{2-} at 1419, 1460 and 1654 cm^{-1} decreases or cease to exist if the calcination temperature rises to 950 °C, possibly due to the decomposition of carbonate to carbon dioxide which can occur at temperatures between 750 and 1100 °C. This result means that lower calcination temperature should be more suitable for preserving the carbonate content [43].

6.4 Concluding Remarks

This chapter reviews fundamental basics in the productions and characterizations of various marine structures before and after conversion to calcium phosphate. The main aim was to concentrate on the inorganic components rather than the organic components of the marine structures.

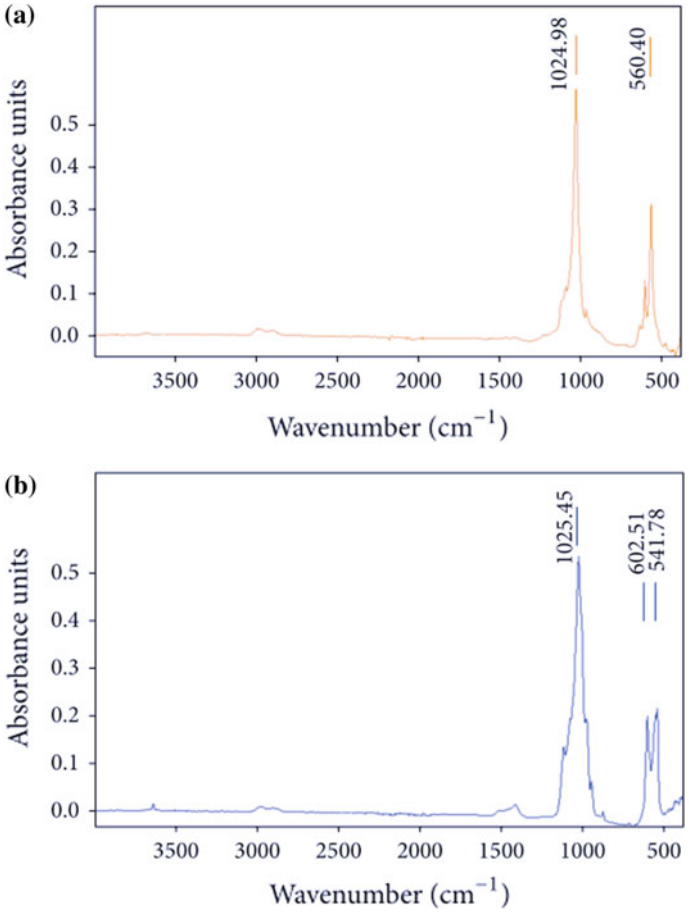

Fig. 6.14 FTIR spectra of shells of *Cerithium vulgatum* powders after calcination for 4 h in air: **a** at 400 °C; and **b** at 800 °C (reprint with permission from [36])

Several examples were given from our research for the following marine structures: fish bone, cuttlefish, corals, sea shells (i.e. limpets, barnacles, sea snails and regular shells), sea urchin, barnacles, and mussels etc. Special emphases were paid to shells and corals as they have been used in the production of HAp and tricalcium phosphate as bone graft materials. The notion of using bone from cuttlefish as a biomaterial, as it has been documented in the literature, were explored in a study by Rocha et al. [2]. Moreover, regular seashells can be collected from uncontaminated beaches, or artificially grown in specially-treated seawater tanks. In the future, there is the probability that none of the sea creatures will remain in our oceans. However, we can collect and preserve dead seashells from the shores for future research. We have summarized several production techniques and characterization methods for

their appropriate conversion for the clinical applications. Here, in those methods, it is also possible to produce nanostructured bioceramics.

Marine-originated biomaterials will continue to supply mankind high purity, well converted and tested biomaterials for orthopedic, dental and maxillo- and craniofacial applications. Their nanostructures will able us to use these materials as slow drug delivery devices and in implant coatings that can deliver antibiotics.

The applications of characterization and testing equipment are constantly improving, and any future research will use these new methods in addition to the techniques explained in this chapter. We hope these new procedures, methods and testing techniques will aid in the development of new implants and devices intended to reduce pain and suffering for the patients and minimize hospitalization time and contribute to our economies.

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Since year 2000 he has published over 200 fully refereed papers in journals, and a book and 43 book chapters. He edited a book on Calcium phosphates and working on a second one on the use of Marine Structures in the Biomedical field. He is the editor of the Journal of the Australasian Ceramic Society. He was awarded by the Australian Ceramic Society's prestigious award for his contributions to the "Ceramics Research & Development and Education in Australia". For his research on multifunctional nanocoatings he also received "The Future Materials Award".

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Chapter 7
Marine-Based Calcium Phosphates from Hard Coral and Calcified Algae for Biomedical Applications

Ipek Karacan, Besim Ben-Nissan and Sutinee Sinutok

Abstract The materials that are developed from the different kind of marine organisms have a broad range of properties and characteristics that can explain their potential functions in the biomedical area. Accordingly, new opportunities are created by biomaterials produced from marine-based sources such as calcium phosphate-based bioceramics, composites, and polymers within the biomedical fields such as new drug delivery systems, the design of novel implantable devices, and various applications in tissue engineering. The major aim of this chapter is to explain the importance of marine structures applicable for biomedical applications as well as choosing the appropriate conversion technique in order to obtain designs and structures best suited for their intended use. Therefore, we will highlight various conversion techniques used in the synthesis of calcium phosphate bioceramics from various marine sources such as *Tubipora musica*, *Foraminifera*, *Porites Hard Corals* and *Halimeda cylindracea* calcified algae, and their biomedical applications in this chapter.

Keywords Hard coral · Calcified algae · Calcium phosphate bioceramics · Tissue engineering · Drug delivery systems

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7.1 Introduction

Over the past four decades, studies on new biomaterials and devices have been expanded progressively. The general definition of a biomaterial is given as "natural or synthetic based non-drug substances which can be used to replace soft or hard tissue in the human body in an effort to improve life quality" [1, 2]. At the moment, researchers have been examining various types of biomaterials-based implantable devices which can be utilized to replace and/or repair different components within the human body. The field of biomaterials employs a multi-disciplinary approach that combines design and selection, materials and clinical sciences. This combination provides the most appropriate biological interaction between the biomaterial and the host tissue, while at the same time restricting any adverse reactions. Therefore, designing and developing any new biomaterial is a long and rigorous process, and it encompasses synthesis, design, manufacturing and so on [3, 4].

Biomaterials, like all materials, are classified into three major categories: metals, polymers, and ceramics. Polymers can be considered as soft materials and their applications are based on soft tissues within the human body such as skin, cartilage, and vascular tissues. The applications of polymers in the medical fields is dependent on their chemical composition and structure such as biodegradability, flexibility, and hydrophilic/hydrophobic ratio [3, 5–7].

On the other hand, metals and ceramics are classed as hard materials and they are utilized in association with hard tissues such as bone tissues. In comparison to polymeric biomaterials, metallic and ceramic-based biomaterials possess greater application opportunities and they have been used in various surgical, orthopedic, cardiovascular and dental applications. Furthermore, they can also be useful in a number of different clinical arenas such as implantable devices, drug delivery systems, and other less common tissue engineering applications [5, 7]. Despite the fact that metallic biomaterials such as titanium and its tertiary alloys such as Ti-6Al-4V have been used in dental and orthopedic applications as implantable devices and prostheses due to their excellent mechanical properties, problems such as corrosion and cytotoxicity caused by metal ion release have been associated with their use. Consequently, these problems may have contributed to an increase in the possibility of implant failure when metallic biomaterials are used [5, 7–9].

In contrast, ceramic-based biomaterials are more attractive than metallic biomaterials owing to their high biocompatibility, high strength and resistance to corrosion. Additionally, bioceramics can be synthesized and engineered with specific properties such as bioactive, bioresorbable, surface-active, or bioinert. Of vital significance is that there are mineral phases which exists in certain bioceramics that are identical to those found in human bone structures. This discovery has subsequently led to an increase in the design and utilization of bioceramics for hard tissue engineering applications such as bone substitution and/or in bone tissue repair and regeneration. Besides tissue engineering applications, bioceramics have also been applied in drug delivery systems as drug carriers [10–12].

Due to the limitations posed by the synthesis method currently used in the manufacture of biomaterials, a new discipline has been developed that focuses on the use of raw materials found naturally. Even though it is referred to as "biomimetics", the field of biomimetics is much wider and usage or imitation of natural materials is only a part of it.

The marine environment is one of the most important sources of material for many biomedical and pharmaceutical applications owing to the vast diversity in the organisms. Especially, the unique architectures and interconnected porous structures of coral and algae skeletons are highly suitable for the drug delivery applications and for hard tissue engineering applications [1, 13, 14].

In this chapter, the production of bioceramics from marine sources, especially hard coral and calcareous algae, and their applications in the tissue engineering and drug delivery systems for hard tissues such as human bone are discussed.

7.2 Bone and Its Structure

Bone tissue can easily repair itself and it has high regenerative capacity compared to other tissues within the human body. Consequently, bone fractures and injuries will heal or recovery naturally without the formation of scars in many cases. In spite of this, bone healing and repair is a long process that usually takes at least 6–8 weeks. At the same time, certain pathological infections can negatively affect the bone healing process [15, 16].

According to "Bone and Joint Decade's Musculoskeletal Health Portal-2018" [17], millions of people worldwide have been suffering from bone and/or joint-related problems such as post-operative inflammations, massive bone defects, osteoporosis, and joint diseases such as osteoarthritis. Furthermore, half of the cases reported concerning bone or joint-related issues have been observed in people who are over 50 years old and/or living in developed countries. More importantly, the percentage of the bone diseases will double by year 2020 according to the latest published work [17].

After a blood transfusion, bone is the second most common tissue that can be transplanted, and it is pertinent that the abovementioned issues such as post-operative inflammation are resolved. An alternative approach is the development of a new generation of bone grafts that is capable of stopping infections in the first place.

On occasions, surgical interventions are necessary to address certain musculoskeletal problems such as osteoporosis, join diseases, bone fractures, infections, skeletal abnormalities, and tumor resections. Hence, biodegradable, temporary, or permanent devices and prostheses have been created by scientists using natural, synthetic or human-derived products [10, 15, 16].

Although implantable bone graft material can support bone healing and substitution, the combination of bone graft with other biological materials such as bone morphogenic proteins can provide osteogenesis, osteoconduction and/or osteoinduction. Allotransplantation, and in particular allograft, utilizes the tissues from one individ-

ual (donor patient) and is transplanted to another patient. Despite the procedure is the most common and have been successfully applied to treat bone-related problems, issues concerning the amount of tissues available for transplantation within our body and the necessity for numerous operations to extract these tissues results in unnecessary pain and suffering to the patients often restricts their utilization. As a result, biomimetic materials obtained synthetically or naturally used for the regeneration of bone tissues is an option which is more attractive than bone tissues derived from humans [15, 16].

Gaining an in-depth understanding into the bone structure is essential to ensure success in the regeneration of both hard and soft tissues using natural or synthetic raw ingredients through the biomimetic approach. The bone structure contains both liquid and solid phases which include micro- and nano-compounds and structures. Principally, human bones can comprised of two highly organized solid phases that have both inorganic and organic components, and so is their architecture which is both unique and complex.

The three major components of bone, which are the mineralized matrix composed of collagen, carbonated apatite phase, and non-collagenous organic proteins, is the reason behind its excellent mechanical properties. Bone tissue is comprised of 35% w/w collagen and 65% w/w carbonated apatite [18, 19]. The organic component, which is the collagen, provides bone with flexibility and at the same time prevents brittleness; while the inorganic components, particularly hydroxyapatite, provides the overall structure and the necessary mechanical properties.

Bone tissue contains two different types of structures which are commonly referred to as trabeculae or cancellous and cortical. The structure of human bone is shown in Fig. 7.1. Cortical bone is dense and hard and is composed of cylindrical shells, whereas trabecular bone is porous and is four times softer than cortical bone. The porosity of trabecular bone is between 50 and 90% depending on the location within the human body (site-specific) [19].

Bone tissue engineering is a highly complex and dynamic process and there are a few important milestones: the first was obtaining a deeper knowledge on the bone remodeling and resorption process; and the second was related to the combined use of several drugs, minerals, bone morphogenic proteins, genes, and growth factors.

Selecting the most appropriate biomaterials for the intended application and treatment option is critical to reduce the chance of failure after implantation. For a specific bone tissue engineering application, the selection process should be centered on factors such as mechanical properties that must be similar to that of the host tissue or that particular location, and an environment that stimulates tissue regrowth after implantation. For that reason, marine-based structures have enormous and valuable potentials in addition to providing highly ideal structures for various tissue engineering applications [18–20].

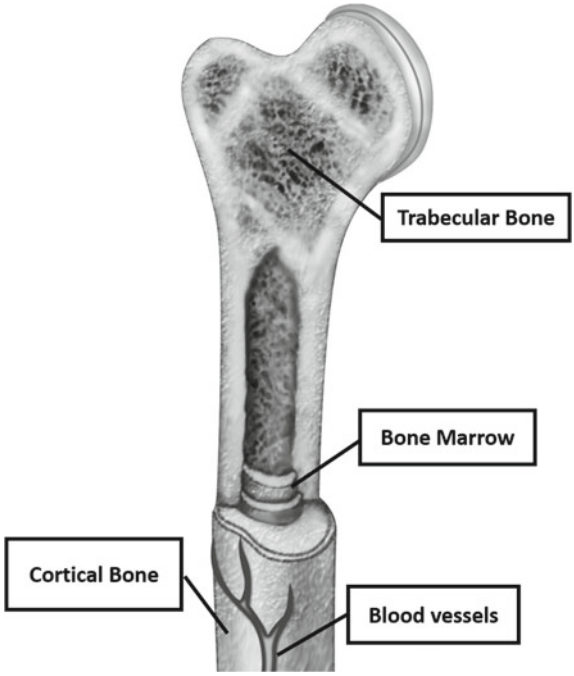

Fig. 7.1 The structure of long human bone

7.3 Marine-Based Calcareous Exoskeletons

Natural biomaterials have been preferred for novel biomedical applications instead of synthetic materials because of their unique biological and structural properties. The most remarkable characteristic of natural marine structures is their amalgamation of inorganic and organic structures, which adds a remarkable property to improve fracture resistance. The common marine structures used in tissue engineering include coral, algae, and sea urchin, which contain both nanoscale inorganic and organic phases. The use of marine-based exoskeleton as calcium carbonate or converting it to hydroxyapatite will allow vascularization and blood supply without any problems due to the presence of natural interconnected pores and channels found within the structure, and ultimately this will permit the growth of new bone.

Marine-based structures are made with immaculate resource and energy efficiency using available materials through self-assembly into highly organized hierarchies. The design of new drug delivery systems and tissue engineering of skeletal tissues using marine-based exoskeletons via the biomimetic approach have yielded promising outcomes [18, 21].

The skeletons of most marine organisms contain calcium carbonate. The calcium carbonate layers are mainly in the form of calcite and aragonite polymorphs. Marine skeletons such as corals have unique architecture and interconnected porous structures, mechanical properties and microstructural composition that are enriched with

bioactive elements to support various functions of human tissues as medical materials. In addition, their pore sizes fall between 20 and 500 μm and values within this range are extremely appropriate for bone cells. Marine-based exoskeletons have been of significance for applications such as tissue regeneration, drug delivery systems, and drug therapy because of these properties and their natural unique structures. Above all, coralline skeletons converted to hydroxyapatite bear a resemblance to the natural architecture, compositions, and characteristics of human bones. Thus, they can replace the human bone structure temporarily while the body regains the ability to heal itself aided by the appropriate degradation rate of the converted hydroxyapatite [22].

On the other hand, calcium carbonate possesses a rapid degradation rate, which is not totally applicable for long-term bone graft applications due to their untimely dissolution and collapse under functional loads. Consequently, converting calcium carbonate to calcium phosphate is necessary as calcium phosphate is more stable than calcium carbonate, and this is of vital importance during the design of long-term bone graft materials and targeted and slow drug release therapies. The conversion process provides improvements in mechanical properties and new chemical composition that allows early bone formation resulted from the release of calcium and phosphate ions from calcium phosphate, which are similar to natural bone minerals [18, 22]. For that reason, a number of studies have been reported regarding the use of micro- or nanosized marine-based materials and bioceramics for a variety of tissue engineering applications and novel pharmaceutical drug delivery devices [21].

7.4 Production of Bioceramics from Marine-Based Sources

Calcium phosphate-based bioceramics have been used in various types of medical hard tissue applications such as orthopedics, dentistry, maxillofacial and cosmetic surgery [23, 24]. In particular, the research and development into marine-derived calcium phosphate have been expanding due to their wide number of clinical applications. Furthermore, a number of studies have reported the use of marine-derived calcium phosphate as drug delivery vehicles because of the unique structures within marine organisms, for example, the interconnected porous structure of hard corals and calcareous algae [25].

Calcium phosphates, whose mineral structures are similar to bone structures, are created using three basic chemical elements: calcium, phosphorus, and oxygen. Consequently, they are defined according to the structure and type of phosphate anions. Hydroxyapatite (HAp), Brushite (DCPD), Monetite (DCPA) and Whitlockite (β-TCP) belongs to the same family of calcium phosphate-based bioceramic materials that are commonly used. More importantly, every calcium phosphate has a unique formula and calcium-to-phosphorus (Ca/P) ratio. For example, the formula and Ca/P ratio for hydroxyapatite is $Ca_{10}(PO_4)_6(OH)_2$ and 1.67, respectively. On the other hand, Whitlockite has a chemical formula of β-$Ca_3(PO_4)_2$ and a Ca/P ratio of 1.50

[26, 27]. Calcium phosphates are also characterized by their bioactivity and resorption properties in addition to their composition and their Ca/P ratio [28, 29].

To date, various techniques used in the synthesis of calcium phosphates have been reported and some of the most commonly used production methods include sol-gel, wet precipitation, hydrolysis methods and solid-state reactions [30]. Nevertheless, issues concerning non-stoichiometric products, long reaction time, uncontrolled particle size and agglomeration have restricted their full utilization. Hydrothermal conversion technique is the preferred approach to synthesize calcium phosphate materials for clinical and engineering applications as better control in the morphology and chemical stoichiometry can be achieved simply by controlling the temperature and pressure used during the conversion process. In addition, cleaning and sterilization of the product is guaranteed with this approach [31, 32].

The production of calcium phosphate bioceramics using natural materials such as those from various marine sources has been the primary focus during the past four decades. This is due to the similarity in terms of physical and chemical characteristics that exists between marine structures and calcium phosphate bioceramics. Hence, it is imperative that the use of marine structures as raw ingredients for biomedical and pharmaceutical applications should be employed resourcefully. Most of the marine structures such as sea shells, corals, marine algae exhibit calcium carbonate ($CaCO_3$) structures, which is the precursor material for various calcium phosphates such as hydroxyapatite [14, 28, 30]. Numerous studies have reported the synthesis of from corals, ostrich eggshells, mollusc, snails, tropical marine algae *Halimeda* spp. using the hydrothermal conversion approach [24, 28, 33].

7.5 Biomedical Applications of Marine-Based Calcium Phosphates

The conversion of various marine structures whose skeletons consist of calcium carbonate such as hard corals and certain types of calcareous algae to calcium phosphates for biomedical applications are highly advantageous as they have highly supportive porous structure and strong mineral arrangements. For over four decades or so, coral and algae exoskeletons have been extensively used as bone grafts, pharmaceutics, and applications in medicine.

In the following sections, advancements in the conversion of different marine structures such as organ pipe red coral, porites hard coral, foraminifera (which is a marine shell), and calcareous algae *Halimeda cylindracea* to calcium phosphate and their applications in tissue engineering and drug delivery systems are discussed.

7.5.1 Tubipora musica

The organ pipe red coral, commonly referred to as *Tubipora musica*, is one of the important potential candidates that can be used for various biomedical applications such as hard tissue engineering and drug delivery systems. The coral belongs to the octo-coral family and composed of the red skeleton and green polyps. Their structure consists of pipe-like vertical tubes of equal size and shape and is connected with one another via horizontal plates [34, 35]. They live in Indo-Pacific and Atlantic coasts and can be maintained artificially with relative ease. More importantly, they can tolerate aquarium conditions. Furthermore, the corals exhibit a solid structure which is ideal for bone tissue engineering [36]. They have been converted from calcium carbonate to various types of calcium phosphates using different conversion methods for various medical applications.

Our previous study has reported that different types of nano-scale calcium phosphates such as hydroxyapatite, tri-calcium phosphate, Whitlockite, and Monetite were observed after the conversion of organ pipe red coral using two different chemical conversion methods which are hot-plate and ultrasonicated conversion methods [37]. According to Fig. 7.2, plate-like calcium phosphate nanoparticles which is mostly Monetite were obtained from the organ pipe red coral that is heat treated after conversion at 400 °C, while spherical-shape calcium phosphate nanoparticles, which is mostly Whitelockite and Hydroxyapatite were obtained after heat treatment at 800 °C.

Agitation rate and temperature treatment can be used to govern the type, particle morphology and size of the calcium phosphate produced. In our research, controlling the morphology and size of coral-derived calcium phosphates provides improvements in the formation of the biomimetic bone scaffold and as a drug carrier for the drug delivery system.

7.5.2 Halimeda cylindracea

Halimeda spp. is a siphonous calcareous green algae and it is a member of the order *Bryopsidales*. They are found in tropical and subtropical reefs around the world such as Atlantic and Indo-Pacific oceans and the Caribbean Sea [38–40]. *Halimeda macroloba* and *Halimeda cylindracea* are some examples of the species of *Halimeda* spp. The structure of these green algae consists of two main components: interconnected plate-like calcified segments and small-uncalcified nodes. The uncalcified nodes provide attachments for calcified segments that create the bushy thallus.

The growth of *Halimeda* involves the formation of a new segment, which begin as white, conical lobes from the apex of the most recent segment, and the white lobe has grown into a complete green segment within 24 h. After 36 h, the calcification of the new segment begins [41]. *Halimeda* spp. precipitates $CaCO_3$ as aragonite into their sediments of the skeleton [38, 42]. The degree of calcification depends

on species, for instance, *Halimeda macroloba* Decaisne is slight to moderate in terms of the degree of calcification in comparison to *Halimeda cylindracea* Decaisne whereas *Halimeda opuntia* (Linnaeus) Lamouroux is more towards the heavy side of calcification. Moreover, older basal segments have a higher amount of calcium carbonate than younger apical segments [41, 43].

In a recent study [33], calcium carbonate in the skeleton of *H. cylindracea* algae was converted to hydroxyapatite using the hydrothermal conversion process under

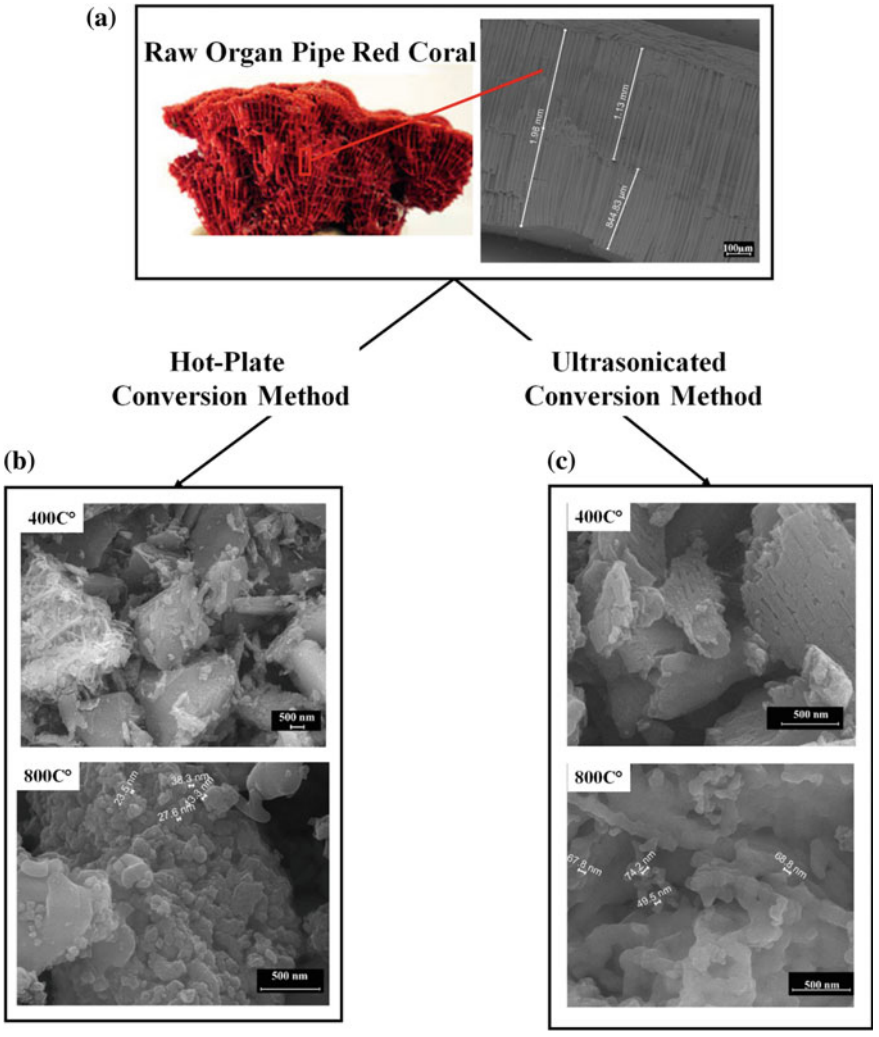

Fig. 7.2 SEM images of organ pipe red coral before conversion (**a**) and after the application of two different conversion methods; hot plate conversion method (**b**) and ultrasonicated conversion method (**c**)

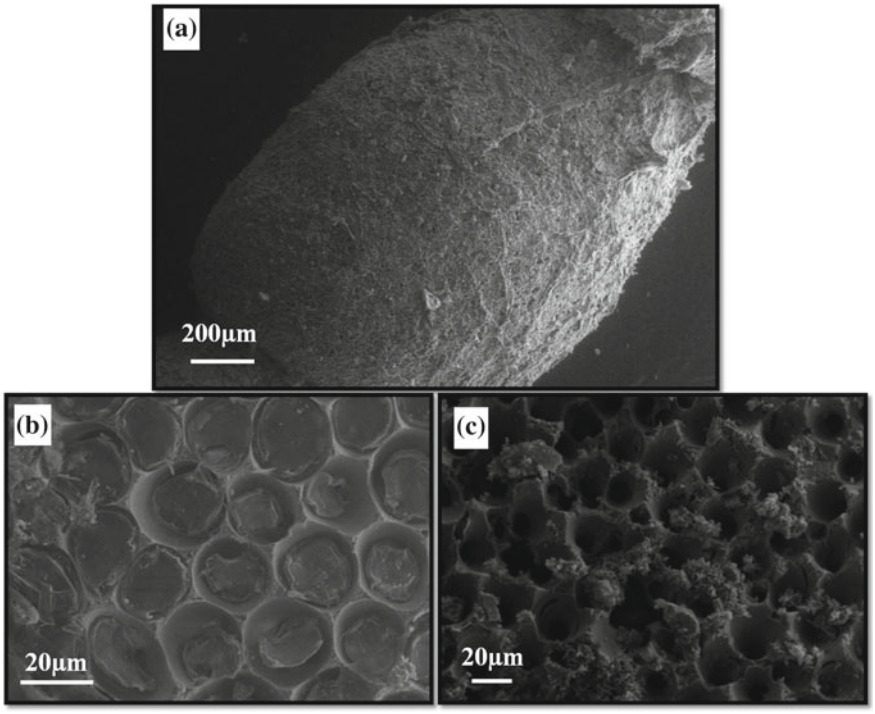

Fig. 7.3 SEM images of *Halimeda cylindracea*. **a** Low and **b** high magnification before hydrothermal conversion. **c** After hydrothermal conversion at high magnification

high pressure and temperature. SEM images of the algae before and after conversion are shown in Fig. 7.3. Because of its fine porous structure, *Halimeda cylindracea* calcified algae are highly suitable for medical applications such as drug delivery systems.

7.5.3 Foraminifera

Foraminifera are single-celled organisms that belong to the family of amoeboid protists. They are important as sediment producers on the coral reefs [44]. Furthermore, foraminifera are often used as environmental indicators for water quality [45] and index fossils for paleoecological research [46].

Foraminifera have reticulating pseudopodia or fine branching strands that merge into a dynamic nets that are used for motility, attachment, shell construction, protection and food trapping [47]. The protoplasm of foraminifera is enclosed by a calcium carbonate shell in a form of magnesium calcite (Mg-calcite) or aragonite [47]. The calcium carbonate shell is developed as one (unilocular) or several (multilocular)

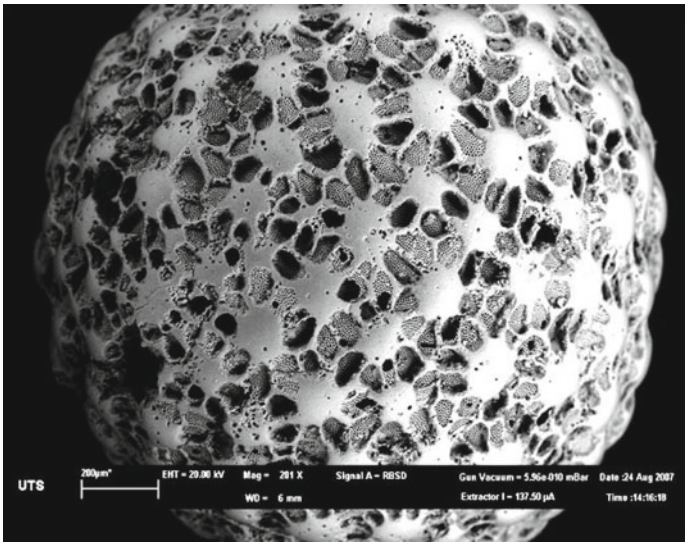

Fig. 7.4 Foraminifera, *Marginopora vertebralis* Quoy and Gaimard, from Heron Island, Great Barrier Reef

chambers depending on the age and species of foraminifera [47, 48]. *Duplella* sp. and *Marginopora vertebralis* Quoy and Gaimard (Fig. 7.4) are some of the examples of unilocular and multilocular species, respectively [47, 48].

Calcium carbonate crystals are precipitated during the calcification process in a direction perpendicular to the shell surface of the outermost layer [49]. The newly formed chambers are relatively thin and slightly calcified. The calcification process will be fully completed within 7–10 days [50]. *M. vertebralis* is symbiotic benthic foraminifera, which houses *dinoflagellate Symbiodinium* sp. The host benthic foraminifera benefit from their algal symbiont by obtaining energy from photosynthesis and enhancing the rate of calcification due to the removal of CO_2 which increase surrounding pH and favors calcium carbonate precipitation [44, 51].

Aragonite or calcite in foraminifera can be hydrothermally converted by chemical replacement of the carbonate with phosphate to produce calcium phosphates that can be used in drug delivery system depending on the Ca/P ratio [47]. Calcium carbonate can be used for a fast-acting drug delivery system, while calcium phosphate such as tri-calcium phosphate is more suitable for a long-term drug release system because calcium carbonate degrades faster than calcium phosphates [47]. β-tricalcium phosphate (β-TCP) derived from foraminifera could be used to carry antibiotics [52], bisphosphonate [53] and simvastatin [54] and used for bone regeneration [55].

Fig. 7.5 Goniopora from Great Barrier Reef Australia

7.5.4 Porites Hard Coral

The Scleractinian corals, *Porites* spp., are reef-building corals commonly found in the tropical and temperate coral reefs around the world such as Atlantic and Indo-Pacific oceans and the Caribbean Sea (Fig. 7.5). They live in compact colonies with many individual polyps that are interconnected by living tissue, feed with tentacles and secrete a calcium carbonate skeleton in the form of aragonite [56]. Corals have endosymbiotic zooxanthellae *Symbiodinium* sp., and they obtain energy from their photosynthetic endosymbionts. Corals that have a large number of zooxanthellae and possess a higher rate of calcification are important contributors to reef growth [56]. The presence of zooxanthellae enhances calcification due to the removal of CO_2 that increase the surrounding pH and favors calcium carbonate precipitation [56].

The calcium carbonate exoskeletons of corals such as *Goniastrea* sp. and *Porites* sp. has been successfully converted to hydroxyapatite using mechanochemical and hydrothermal techniques for biomedical applications such as drug carrier and bone substitution biomaterial due to their osteoconductivity, biocompatibility, and it is relatively safe to use [25, 33, 57, 58]. Some of their unique features include the amount of porosity and the size of their closed pores. The amount of interconnecting pores and their sizes recorded in *Porites* sp. are approximately 40–60% and between 100 and 500 μm, respectively. As far as their interconnectivity and sizes are concerned, it is similar to human cortical bone and osteon found in human bone [57].

Calcium carbonate skeleton of *Porites* sp. was converted to plate-like shaped hydroxyapatite crystal by hydrothermal conversion method under high pressure and temperature [33]. Figure 7.6 shows the SEM images of Porites before and after the

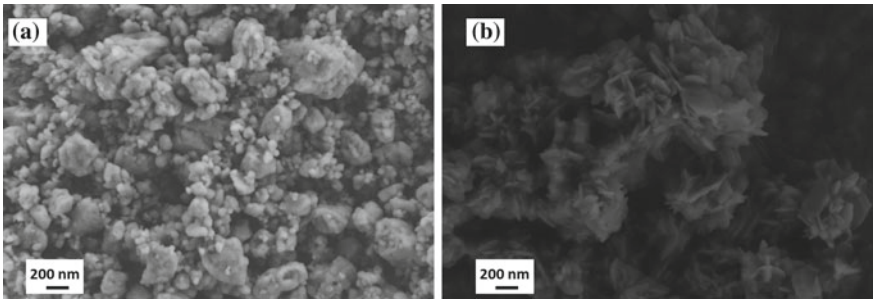

Fig. 7.6 SEM image of cleaned and unconverted *Porites* powder (**a**) and converted *Porites* powder (**b**)

conversion process. It was suggested that *Porites* is highly applicable to long-term long bone orthopedic implants and as long-term slow and sustained drug delivery system.

Due to the specific nano- and meso-porous structure of *Porites* hard coral, they have been utilized as a drug carrier once it is converted to calcium phosphate using appropriate techniques. Macha et al. reported that hydroxyapatite particles that were converted from Porites hard coral have been used as a delivery vehicle in an effort to regulate the rate of release of a drug delivery system [59]. The release rate of bisphosphonate, a drug is that is able to reduce bone resorption, was successfully controlled when it was loaded onto coral-based hydroxyapatite particles. Furthermore, coral-based hydroxyapatite has been applied as a drug delivery vehicle in implantable systems for dental and orthopedic applications [60]. Particles were loaded with the antibiotic gentamicin and deposited onto the surfaces of metallic implants to prevent implant-related infections. The study found that the use of coral-based hydroxyapatite particles was able to control the speed of drug release, and ultimately it can be applied as a slow drug delivery system to assist in the healing process after implantation.

7.6 Concluding Remarks

Porites hard coral, Foraminifera, *Halimeda cylindracea* and *Tubipora musica* are excellent example of marine-based sources that can be used to convert to calcium phosphate that can be utilized in various biomedical applications such as implantable medical devices or in the drug delivery systems.

As a result of their high biocompatibility, osteoconductivity and structural similarity to human bone, calcium phosphate derived from various marine sources have been applied as implantable medical devices such as bone grafts. In addition, they have a unique natural porous structure which is highly suitable for a drug delivery system as carrier materials. In conclusion, using natural marine-based sources increases to the likelihood of obtaining successful results for biomedical applications.

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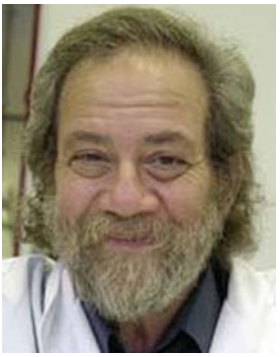

Besim Ben-Nissan Prof. Ben-Nissan has B.Eng. in Metallurgyical Engineering (ITU), M.Sc. degree in Ceramic Engineering and Ph.D. in Mechanical and Biomedical Engineering both from the UNSW Australia.

During in his formative years Prof. Ben-Nissan worked on Titanium and its alloys and Magnesium alloy development and casting technologies and their properties for both engineering and medical applications. Over the last four decades, Professor Ben-Nissan has worked and contributed to the biomedical materials, implant design, production and analysis of various advanced ceramics, nano-coated sol-gel developed thin films, coated orthopedic and dental implants, anti-microbial slow drug delivery devices and methods, marine structures for clinical applications, biomechanics and finite element analysis of medical materials and engineering structures.

He has successfully developed materials for implant technology such as ceramic knee prosthesis, calcium phosphate based bioactive materials, bone graft production and bio-composites, and conducted research on biomechanics and modelling such as jaw bone, knee and hip joints, reliability and implant design modular zirconia ceramic knee prosthesis, femoral head and taper stresses and artificial ocular implants and bionic eye and recently on 3D printing of bioceramics and metallic implants and anti-microbial multifunctional coatings for drug delivery which are supported by the European Commission and the Australian Academy of Science research grants.

Since year 2000 he has published over 200 fully refereed papers in journals, and a book and 43 book chapters. He edited a book on Calcium phosphates and working on a second one on the use of Marine Structures in the Biomedical field. He is the editor of the Journal of the Australasian Ceramic Society. He was awarded by the Australian Ceramic Society's prestigious award for his contributions to the "Ceramics Research & Development and Education in Australia". For his research on multifunctional nanocoatings he also received "The Future Materials Award".

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Part II
Marine Sources for Drug Delivery

Chapter 8
Application of Chitosan Based Scaffolds for Drug Delivery and Tissue Engineering in Dentistry

Sevda Şenel, Eda Ayşe Aksoy and Gülçin Akca

Abstract Chitosan is a marine polymer, which possesses numerous favorable properties including bioadhesivity, biodegradability and biocompatibility, which have enabled its use in drug delivery and tissue engineering. Furthermore, chitosan has been widely investigated in vitro and in vivo for its bioactive properties such as anti-inflammatory, antimicrobial, hemostatic, wound healing etc. This chapter will comprehensively detail the promising characteristics of chitosan as a biomaterial for drug delivery and tissue engineering, with regard to its safety, quality and efficacy, and review the recent advances on its applications in dentistry.

Keywords Chitosan · Oral cavity · Anti-inflammatory · Antimicrobial · Periodontitis · Dental disease · Caries

8.1 Introduction

The oral cavity is the first section of the digestive system and is delineated by the lips, cheeks (buccal), hard palate, soft palate and floor of mouth (sublingual). It also consists of teeth, gingiva (gum) and their supportive tissues (Fig. 8.1).

The oral cavity contains some of the most varied and vast flora in the entire human body and is the main entrance for gastrointestinal and respiratory systems, which are

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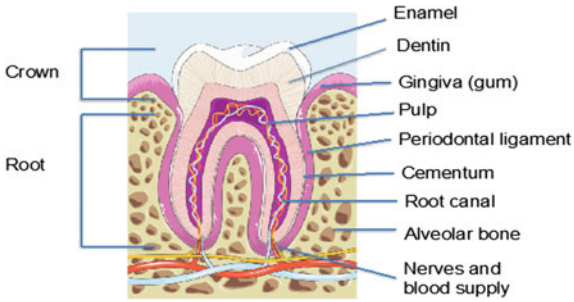


Fig. 8.1 Schematic illustration of anatomy of tooth. (Modified from smart.servier.com)

vital to human function and physiology. Several diseases involve these two systems and manifest in the oral cavity.

In general, oral health is regarded as an essential component of general health. Links between common oral infections and serious general health conditions including malnutrition, alcoholism, diabetes, cystic fibrosis, renal failure, heart failure, and chronic lung disease is well-recognized [1–3]. Therefore, it is very important to maintain the health of the oral cavity. Odontogenic infections, such as periodontitis, dental caries, endodontic infections, pericoronitis and dental abscesses, and non-odontogenic infections (in which teeth are not involved) such as oral trauma, radiation injury, chemotherapy mucositis, salivary gland infection, lymph node abscess, and postoperative infection, are the most common acquired problems in the oral cavity.

In general, non-surgical treatments such as scaling and root planning and administration of therapeutic agents are applied to treat the diseases in the oral cavity. In advanced cases, surgical treatment approaches such as root canal treatments, extraction of the teeth, surgical interventions, and prosthetic appliances are applied for treatment of these disease states. However, in many cases, these treatments may not be enough for the recovery and local treatment approaches such as local delivery of antibiotic, analgesic or anti-inflammatory drugs have been applied in conjunction with other treatments [4–10].

Numerous innovative drug delivery systems have been developed for the local treatment and prevention of various diseases in the oral cavity. However, there are still few systems available on the market, and many challenges still remain to overcome, including removal of the system from the site of action due to salivation as well as physiological functions such as eating, drinking, and swallowing or flushed by saliva and the disappearance of its effect. On the other hand, tissue engineering has been an attractive and effective approach to repair and restore function of damaged or diseased hard and soft tissues in the oral cavity [11–17]. Amongst the approaches that have been considered in tissue engineering, the most common approach is utilization of biodegradable materials to build a scaffold [18–20].

Various natural and synthetic polymeric systems have been used for drug delivery as well as tissue regeneration in treatment of oral diseases. Amongst them is the marine polymer chitosan, which exerts favorable features such as biocompatibility,

biodegradability and bioadhesivity. Chitosan has been widely investigated in dentistry during the last two decades not only as a drug delivery system but also as a tissue engineering material and as a therapeutic agent due to its bioactive properties such as antimicrobial, hemostatic, anti-inflammatory, wound healing etc. This chapter will give an overview of the recent progress in applications of chitosan in dentistry as well as our experiences we have gained in this field in the last two decades.

8.2 Local Treatment in Dentistry and Chitosan

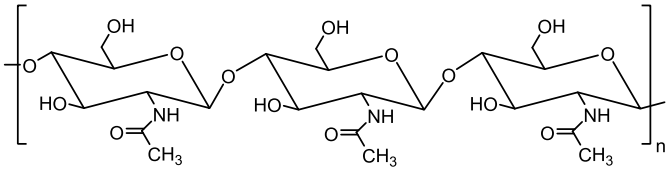
In late 90s, the role of local antibiotic delivery systems in the management of periodontal diseases was recognized, which resulted in a shift in treatment modalities of dental diseases [21]. The antimicrobial resistance, undesired effects due to systemic administration of antibiotics has further advocated the use of local delivery of drugs into the periodontal pocket. Amongst the first drug delivery systems brought to the market were chlorhexidine chip [22], metronidazol oral gel [23], minocycline dental gel [24] and doxycycline polymer [25].

Success of a local drug delivery system into oral cavity depends on its ability to deliver the drug to the application site for required period and provide longer retention time for enhanced therapeutic efficacy. Delivery systems based on bioadhesive polymers have been widely investigated to avoid the removal of the delivery system from the side of application due to saliva flow and tongue movement. These polymers help the system remain attached on the application site and provide prolonged release of drug. Marine polymers such as alginates and chitosan have been excellent candidates for this purpose due to their biocompatibility, bioadhesivity and biodegradability [26–29].

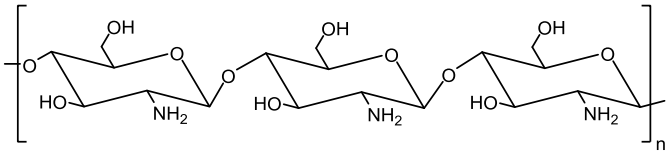
Chitosan is a cationic polysaccharide obtained by deacetylation of chitin, which is naturally available in shells of crustaceans such as crabs and shrimps. In recent years, the production of chitosan from fungal sources has gained increased attention due to several advantages in terms of homogenous polymer length, high degree of deacetylation and solubility over the marine sources [30].

Chitosan has a linear structure consisting of ß (1-4) linked D-glucosamine with randomly located N-acetylglucosamine groups depending upon the degree of deacetylation of the polymer (Fig. 8.2). The degree of deacetylation represents the proportion of N-acetyl-D-glucosamine units with respect to the total number of units, which can be used to differentiate between chitin and chitosan [28]. Chitin with a degree of deacetylation of 65–70% or above is generally known as chitosan. Deacetylation can be randomized or block wise (Fig. 8.2). The degree of deacetylation and deacetylation pattern defines the physicochemical and biological properties of chitosan. Accordingly, it is possible to tailor chitosans with desired properties.

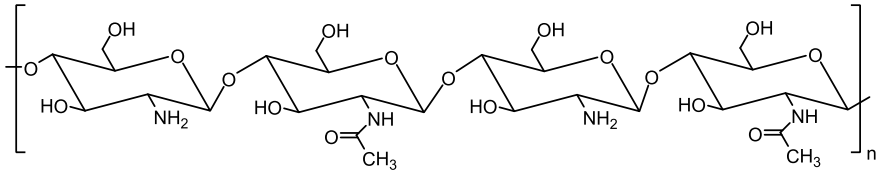
For medical applications, chitosan is preferred over chitin due to its superior solubility and its free amine groups, which are active sites for chemical reactions to construct sophisticated molecular architectures with enhanced activities. Chitosan is readily soluble in dilute acidic solutions below pH 6.0, and amino groups become



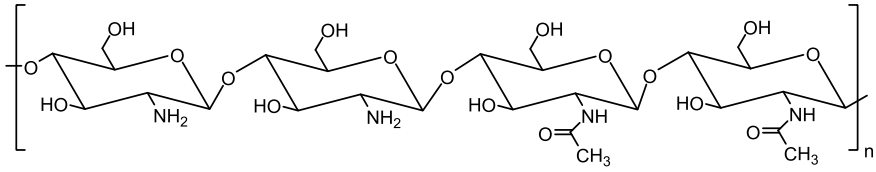
(a) Chitin (100 % acetylated)



(b) Chitosan (100 % deacetylated)



(c) Chitosan (partially deacetylated-randomised)



(d) Chitosan (partially deacetylated-blockwise)

Fig. 8.2 Chemical structure of chitin and chitosan

deprotonated and the polymer loses its charge and becomes insoluble with the increasing pH. The solubility of chitosan is dependent on the degree of deacetylation and the method of deacetylation used since the pKa value is highly dependent on the degree of N-acetylation [31].

The positive surface charge of chitosan allows it to interact with macromolecules like exogenous nucleic acids, negatively charged mucosal surfaces, or even the plasma membrane [32, 33]. Chitosan is degraded by enzymes such as chitosanase

and lysozyme. The key factors for controlling the biodegradation rates of chitosan are the degree of deacetylation and molecular weight as well as its crystallinity [34, 35]. Modified chitosans were reported to exert higher biodegradation owing to the destroyed crystalline structure of chitosan [36]. Chitosans with degrees of polymerization smaller than 20 and with an average molecular weight less than 3900 Da are called chitosan oligomers, chitooligomers, or chitooligosaccharides [37]. Chitooligosaccharides are the obtained by enzymatic or chemical hydrolysis of chitosan. Due to their higher solubility and enhanced bioactive properties, chitooligosaccharides have been widely investigated for biomedical applications [38].

The cationic character of chitosan, which is due to its primary amino groups differs it from other marine polysaccharides. These primary amino groups are responsible for properties such as prolonged drug release, gel-forming capability, mucoadhesion, penetration enhancement, etc. It is possible to improve these properties by chemical modifications. The chemical structure and relevant biological properties of chitosan highlight the suitability and extensive applications of chitosan in dentistry. Within the last two decades, a considerable amount of work has been published on application of chitosan and its derivatives in dentistry [39–41].

8.3 Applications of Chitosan in Dental Diseases

Chitosan has been a very attractive material which has been extensively investigated in dental field both for its bioactive properties such as wound healing, tissue regeneration, antimicrobial and as a safe material for delivery of drugs, especially the anti-inflammatory and antimicrobial molecules. The brevity of the current chapter precludes the comprehensive review of the applications of chitosan in all dental diseases mentioned above; hence, the authors will mainly focus on antimicrobial activity and tissue engineering role of chitosan.

8.3.1 Antimicrobial Activity and Delivery of Antimicrobial Drugs

Chitosan exerts antimicrobial activity against a wide range of microorganisms, including the Gram-negative and Gram-positive bacteria and fungi. In general, the antimicrobial activity of chitosan is commonly explained by the interaction between positively charged chitosan molecules and negatively charged microbial cell membranes. This interaction is mediated by the electrostatic forces between the protonated amino groups and the negative residues. Yet, the results reported on antimicrobial activity and chitosan characteristics show variations, which is mostly attributed to the differences in properties of chitosan, such as source, type, molecular weight, degree of deacetylation, viscosity, solvent and concentration [42–44]. It also depends

on the strains of the microorganisms tested. Furthermore, it has been shown that environmental effects such as pH, temperature, salinity play a significant role in its antimicrobial activity [45, 46].

Chitosan has been used for its antimicrobial activity in treatment of various oral infections, such as dental caries, periodontitis, etc. Dental caries is the most prevalent and worldwide oral disease that affects a significant proportion of the world population. The development of dental caries primarily involves *Lactobacilli spp.* and *Streptococcus mutans*. Chitosan been demonstrated to exert antibacterial activity against *S. mutans* and *Lactobacilli spp.*, therefore can reduce dental caries in gel, paste, solution and chewing gum forms [47–51].

In addition to its own antimicrobial, chitosan has been successfully applied for local delivery of numerous antimicrobial drugs such as metronidazole, moxifloxacin, chlorhexidine gluconate, and antiviral drugs such as acyclovir etc. used in treatment of periodontal and oral mucosal infections, providing an improved efficiency [5, 44, 52–54].

It is possible to prepare chitosan-based delivery systems in different dosage forms such as gels, films, fibers, tablets, sponges, micro/nanoparticles, etc. Due to ease of application by patient, in most cases the gel and film forms are preferred. Furthermore, due to bioadhesive properties, the formulation remains longer on the application site providing higher drug penetration, efficacy and acceptability, with reduced dosing intervals.

In one of our previous studies, we have investigated the antimicrobial activity of chitosan formulations both in gel or film form against a periodontal pathogen, *Porphyromonas gingivalis* [52]. In this study, we have shown that chitosan exhibited antimicrobial activity against the tested strain and the combination of chitosan with chlorhexidine gluconate showed a higher activity when compared to that of chlorhexidine gluconate alone, which would provide chlorhexidine gluconate application at lower concentrations thus avoiding its unwanted side effects. Later, we have investigated the chitosan in film form alone or incorporated with chlorhexidine gluconate as alternative tissue-conditioning systems in patients who have undergone sulcoplasty surgeries for oral rehabilitation, in comparison to a commercially available tissue-conditioning agent, Visco-gel® [55]. The films were applied only on one-half of the inner side of the splints, then the splints were fixated onto operation site. Antimicrobial activity against *Candida species (spp)*, *Staphylococcus spp*, *Streptococcus spp*, *Diphteroid basilles*, *Escherichia coli, and Neisseria spp* was measured on the samples taken from the surface of the healing tissue and the surgical splints on post-operative days. A significant decrease was observed in colonization of pathogens on both the healing tissue and the surgical splint after application of the chitosan formulations.

With its bioadhesive and antimicrobial property, chitosan was suggested as an excellent candidate for tissue conditioning. Furthermore, chitosan film was able to protect the ulcerated areas of the mucosa by moistening and coating it. We have recently compared the antimicrobial activity of chitosans with different properties (molecular weight, solubility) and obtained from animal (crab shell) or non-animal

(white mushroom) against various three major oral pathogens, *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans , and Candida albicans* [44].

The antimicrobial activity was found to increase with the increased molecular weight of chitosan. Water-soluble chitosan was found to be more effective when compared to base chitosan gels. However, it is difficult to make a generalization as the results reported on antimicrobial activity of chitosans has shown differences in regard to correlation between the activity and molecular weight, solubility, degree of deacetylation.

This variation in antimicrobial activity of chitosans can be attributed to differing degrees of affinity between the cell walls of the bacteria and the chitosan, and to different degrees of deacetylation of the chitosan. Furthermore, the environmental conditions such as pH, the nature of the bacteria and fungi affect the efficiency of the system. Therefore, for applications in treatment of local infections both as a delivery system and an antimicrobial agent, the selection of the source, molecular weight and solubility properties of chitosan is very important to obtain the desired effect in regard to antimicrobial activity, drug release and bioadhesion.

8.3.2 Tissue Engineering

The dental diseases such as caries and periodontitis can result in a permanent loss of tissues and functions, thus affect the daily life of populations in all age groups worldwide. Those who have missing teeth and diseased tissues would have difficulties in eating and speaking; which ultimately leads to a reduction in the life quality of the patient. Furthermore, such conditions can result in emotional problems due to their unhealthy appearance and feelings. Tissue engineering using biomaterials has gained great interest in biologic regeneration of damaged dental tissues as an alternative to current clinical treatments. Regeneration of whole tooth as well as dentin, pulp, and periodontal ligament are important targets for dental tissue engineering [56–58].

In dental tissue engineering, scaffold-free approaches such as cell therapies, cell sheets and micro-tissue are used for regeneration of new tissues, and scaffold-based approaches have been applied to provide an appropriate environment for cell attachment and proliferation for further regeneration of the tissue. Regardless of the tissue type, biocompatibility, biodegradability and mechanical properties are the key requirements in developing such scaffolds [59, 60]. They have to provide cell attachment without disturbing normal cell functionality and cell proliferation, and should not show inflammatory response and exhibit appropriate biodegradation period during new tissue regeneration. Their biodegradation products should also be safe and non-toxic. More important, the scaffold should have an appropriate mechanical property. Mechanically stable scaffolds allow handling, adaptation and integration in the defected tissue site [59]. These scaffolds produce an artificial environment suitable for cell adhesion, migration and proliferation along with synthesis of extracellular matrix (ECM).

Material selection is one of the most critical issues in achieving a successful scaffold for tissue regeneration in dentistry. Numerous natural and synthetic biomaterials have been investigated to develop tissue scaffolds. With synthetic polymers, the composition and properties of the scaffolds can be easily controlled but most of them are not biodegradable and a second surgical procedure is needed to remove them, whereas with natural polymers, due to their biodegradable and bioactive properties better performance is obtained in biological systems [61].

As a scaffold biomaterial, chitosan exerts numerous benefits in regard to its chemical composition (which is similar to glycosaminoglycans), molecular weight, solubility, surface charge, water absorption capacity, biodegradability, biocompatibility [62, 63]. Furthermore, versatility in its surface chemistry as well as its biological properties makes it very attractive in tissue engineering applications.

In order to develop chitosan scaffolds with improved mechanical strength and tunable topography, numerous strategies have been applied such as chemical crosslinking and/or formation of composites with reinforcement agents, synthetic or natural polymers. It is important that such applications to improve the mechanical properties of chitosan scaffolds should not alter the biological properties of the scaffold, and not cause any unfavorable toxic effects on cells. It has been demonstrated that, mechanical strength can be improved also by altering the properties of chitosan such as ionic strength, solubility [64].

Numerous methods have been applied for fabrication of highly porous 3D chitosan scaffolds such as freeze-drying, electrospinning, salt-leaching, thermally induced phase separation, 3D-printing, layer by layer assembly [65–69].

Chitosan scaffolds with interconnecting pores of appropriate dimensions can be produced in hydrogel form or in solid state in various forms such as films, fibers, sponges, microparticles [62, 70–72].

The degree of deacetylation and molecular weight of the chitosan has been shown to have significant impact on biological properties of the scaffold. Molecular weight of chitosan ranges between 100 and 1000 kD, and it is inversely proportional to degree of deacetylation. With higher molecular weight, its viscosity is increased, which would also have an impact on tissue interaction. Degradation of chitosan was observed to delay with increased degree of deacetylation (above 85%). In monographs of chitosan included in European Pharmacopeia [73] and United States Pharmacopeia [74], the degree of deacetylation is stated above 70%. In general, the commercially available preparations have a degree of deacetylation between 60 and 95%.

The degree of deacetylation has been shown to play a key role in cell adhesion and proliferation [75]. Domard and his colleagues [76] showed that chitosan films were cytocompatible towards keratinocytes and fibroblasts regardless of the degree of deacetylation. They also demonstrated that the higher the degree of deacetylation of chitosan, the lower was the cell adhesion on the films. Keratinocyte proliferation was found to increase when the degree of deacetylation of chitosan was decreased, indicating that the degree of deacetylation influences the cell growth in the same way as cell adhesion. On the other hand, it was observed that although they remain alive,

fibroblasts did not proliferate on chitosan films. This behavior was attributed to the extremely high adhesion, which certainly inhibits cell growth.

Previously, we have also studied the effect of chitosan on osteoblast and fibroblast cell attachment using base and water-soluble chitosan with degree of deacetylation of 70% [77]. Our results suggested that both the water-soluble and water-base chitosan supported the initial attachment and spreading of osteoblasts preferentially over fibroblasts, which would be beneficial in restoring bone deficiencies by favoring the early attachment and proliferation of osteoblasts over fibroblasts. In another study, normal human osteoblast precursor cell attachment and proliferation was investigated on series of films prepared using seven different chitosans (76–96 degree of deacetylation and MWt = 2400–8200 kDa) [78]. No trend or correlation between the degree of deacetylation, crystallinity, contact angle, molecular weight, residual ash or protein content and the attachment and growth of bone cells on chitosan films was observed. Such divergent results obtained for the degree of deacetylation and cell attachment and growth can be attributed to the differences in origin of the chitosans used as well as their process conditions and the procedures applied during the cell culture studies.

8.3.3 Treatment of Periodontitis

Periodontitis, which is considered to be the second most common dental disease worldwide, after dental caries, is a common chronic infectious disease characterized by destruction of surrounding structures of teeth resulting in alveolar bone loss, periodontal ligament destruction, gingival recession, bone loss in the furcation area, increased tooth mobility and eventually leading to tooth loss [79].

In order to restore the homeostatic relationship between periodontal tissue and its polymicrobial dental-plaque community, prevention and treatment is applied to control the bacterial biofilm and other risk factors, and restoring lost tooth support. The most widely used treatment is physical removal of the plaque by scaling and root planning. Local or systemic delivery of antimicrobial and anti-inflammatory drugs also helps to control the infection.

In advanced periodontitis, regenerative surgical procedures using grafting materials, guided tissue regeneration, and the use of enamel matrix derivatives (EMD) in a clinical setting. Furthermore, stem cell therapies, recombinant human growth factor therapies and their combinations in matrix-based scaffolds have been applied to regrow, repair or replace damaged cells and tissues [18, 19, 80–82].

Numerous chitosan-based delivery systems and scaffolds have been developed for local treatment of periodontitis. Chitosan itself has been demonstrated to inhibit the growth of periodontal pathogens and modulate the inflammatory response in periodontitis [83–88]. Previously, we have studied the effect of chitosan gel incorporated with 15% metronidazole, which was applied adjunctive to scaling and root planning in patients with chronic periodontitis [5]. In presence of chitosan, significant improvements were observed in clinical parameters such as probing

depth (PD), clinical attachment level, the amount of gingival recession, plaque index, gingival index, and gingival bleeding time index. Later, we have investigated the effect of chitosan alone and its combination with collagen membrane as well as with demineralized bone matrix on periodontal regeneration applied into the intraosseous lesions in patients with chronic periodontitis. Clinical and radiographic measurements demonstrated that chitosan gel as well as its combination with demineralized bone matrix or collagen membrane was effective in healing of periodontal lesions [13]. This study was one of the first studies evaluating chitosan gel in a periodontal surgery in humans.

Recently, we have prepared gel formulations of chitosan in combination with a statin group drug, atorvastatin, which has been reported to exert anti-inflammatory effect in periodontal diseases [89]. The anti-inflammatory effect of the locally delivered chitosan formulations was evaluated in rats with ligature induced periodontitis. A decrease in release of pro-inflammatory cytokines (IL-1β, IL-6 and IL-8) and anti-inflammatory cytokines (TGF-β1 and TGF-β2) and a significant alveolar bone healing was observed. The anti-inflammatory activity was found to enhance in presence of chitosan. No difference was observed between base and water-soluble chitosan.

Since 1980s, guided tissue regeneration has been successfully applied in periodontal tissue regeneration to stop cell migration from gingival connective tissue and epithelium to the periodontal defect [90, 91]. Guided tissue regeneration is defined as a surgical procedure with the goal of achieving new bone, cementum, and periodontal ligament attachment to a periodontally diseased tooth, using barrier devices or membranes to provide space maintenance, epithelial exclusion, and wound stabilization [92]. Subsequently, a membrane technique used to generate new bone around implants based on the principle of guided tissue regeneration was defined as guided bone regeneration (GBR) [93].

Biodegradable natural or synthetic polymeric membranes have been widely used in guided tissue regeneration and guided bone regeneration [94]. Due to its promising properties emphasized in previous sections, chitosan has been one of the most investigated biopolymers applied in guided tissue regeneration and guided bone regeneration. However, chitosan alone was found not to be sufficiently bioactive to stimulate optimum bone tissue regeneration in guided tissue regeneration, therefore it has been mostly investigated in combination with other materials such collagen, coral, sodium hyaluronate as well as therapeutic agents [39, 62, 72, 86, 95–100]. The recent studies on applications of chitosan in dental tissue regeneration are summarized in Table 8.1. Due to the large number of studies reported in this field, only the recent studies were given.

8.4 Conclusion and Future Perspectives

Due to its bioadhesive, biodegradable and bioactive properties such as anti-inflammatory, antimicrobial and hemostatic as well as its structural similarity to naturally occurring GAGs, chitosan is regarded as a very promising biomaterial in

Table 8.1 Studies performed on development of chitosan-based scaffolds in dental tissue engineering in the last 5 years

Scaffold type	Chitosan type*	Other ingredients	Aim	Application	Result	Refs.
Sponge	Chitosan Av Mw: 370 kDa Degree of deacetylation: 75–85% Sigma-Aldrich	Gelatin Bioactive glass	Bone regeneration	In vitro—dental pulp cells In vivo—critical-sized femoral defect model in rat	Osteogenic differentiation with high alkaline phosphatase activity New bone formation (80%) in the defect area eight weeks after implantation	101
Sponge	Chitosan Av Mw: 370 kDa Degree of deacetylation: 75–85% Sigma-Aldrich	Simvastatin	Pulp dentin regeneration	In vitro—odontoblastic phenotype dental pulp cells	Chemotactic and bioactive effects on dental pulp cells	102
Trilayer sponge	Chitosan Medium Mw Low Mw		Periodontal regeneration (bone and gingiva)	In vitro—gingival fibroblasts, human osteoblasts, human PDL fibroblasts In vivo—periodontal ectopic model in nude mice	Adherence and elongation of all cells types Tissue ingrowth, and vascularization	103
Sponge	Chitosan Av Mw: 25 kDa Degree of deacetylation: 80% Mian Scientific Company	Hydroxypropyl methylcellulose Bioactive glass Zinc oxide	Alveolar bone repair Antibacterial activity	In vitro—MC3T3-E1 mouse pre-osteoblasts Antibacterial activity against *S. aureus*	Enhanced cell growth, adhesion, proliferation and differentiation Inhibition of *S. aureus* growth	104

(continued)

Table 8.1 (continued)

Scaffold type	Chitosan type*	Other ingredients	Aim	Application	Result	Refs.
Sponge	Chitosan Mw: 190–375 kDa Degree of deacetylation: 85% Sigma-Aldrich	Alginate PLGA	Periodontal regeneration	In vitro—cementoblasts	Increased cell proliferation, differentiation, mineralization	105
Sponge	Chitosan Medium Mw Degree of deacetylation: 75–85% Sigma-Aldrich	Arginine-glycine-aspartic acid (RGD) Fibronectin	Stem cell based regeneration	In vitro—human dental pulp stem cells	Supported cell attachment and proliferation, but not odontogenic differentiation	106
Sponge	Chitosan Degree of deacetylation: 75–85% Sigma-Aldrich	Collagen Calcium aluminate	Evaluation of odontogenic potential	In vitro—human dental pulp cells	Induced intense migration of human dental pulp cells, with cellsattaching to and spreading on the material surface	107
Sponge	Chitosan Degree of deacetylation: 75–85% Sigma-Aldrich	Hyaluronic acid	Periodontium regeneration	In vitro—NIH3T3 and MG63 cells	High CD44 expression and cell migration	108

(continued)

Table 8.1 (continued)

Scaffold type	Chitosan type*	Other ingredients	Aim	Application	Result	Refs.
Sponge	Chitosan Av Mw: 634 kDa Degree of deacetylation: 89% Hunza Nutriceuticals	Transforming growth factor-ß1	Osteogenic differentiation of dental pulp stem cells	In vitro—multipotent stem cells derived from human exfoliated deciduous teeth (SHED)	Enhanced proliferation and osteogenic differentiation of SHED	109
Sponge containing nanofiber	Chitosan Sigma-Aldrich	Polycaprolactone-Poly(ethylene glycol)	Periodontium regeneration	In vitro—rat bone marrow mesenchymal stem cells (rBMSCs)	Oriented arrangement and ligamentogenesis of rBMSCs	110
Bilayered construct: nanofiber membrane and sponge	Chitosan Mw: 100–150 kDa, Degree of deacetylation: 85% Koyo Chemical	Polycaprolactone Calcium sulphate	Regeneration of alveolar bone and periodontal ligament	In vitro—human dental follicle stem cells (hDFCs)	Enhanced attachment, infiltration, proliferation, and differentiation of stem cells to osteoblast and fibroblast cells	111
Nanofiber	Chitosan	Polyhydroxybutyrate Nanobioglass	Pulp-dentin regeneration	In vitro—stem cells harvested from human deciduous dental pulp	Growth and proliferation of human dental pulp stem cells is supported	112
Nanofibrous membrane	Chitosan Av Mw: 370 kDa Degree of deacetylation: 75–85% Sigma-Aldrich	Polyurethane Ag nanoparticle	Dental barrier membrane Antibacterial effect	In vitro—fibroblast cell line Antibacterial study against *P gingivalis*	Cell viability decreased to 80% with increased Ag nanoparticle content Inhibition of growth zone increased with increasing concentration of Ag nanoparticle	113

(continued)

Table 8.1 (continued)

Scaffold type	Chitosan type*	Other ingredients	Aim	Application	Result	Refs.
Nanofiber Membrane	Chitosan Mw: 3115 KDa Degree of deacetylation: 71%	Triethylamine/di-tert-butyl dicarbonate (TEA/tBOC)	Guided bone regeneration	In vitro—osteoblast In vivo—calvarial defects in rats	High cellular viability of osteoblasts at day 5 Prevented soft tissue infiltration and growth of new bone	114
Bilayered membrane	Chitosan Mw: 90–310 kDa Degree of deacetylation: 85% Fluka	Collagen	Periodontal guided bone regeneration	In vitro—mesenchymal stem cells In vivo—calvarial defects in white rabbits	Proliferation, metabolic activity and osteogenic gene expression New bone formation in defect site without inflammation	115
Nanofibrous mats and films	Chitosan Av Mw: 146 g/mol Degree of deacetylation: 83%, Synthesized from shrimp waste	Piroxicam Poly(vinyl alcohol) Hydroxyapatite	Periodontal regeneration	In vitro—VERO cell line	Higher cell proliferation on fibrous mats compared to that on films	116
Nanoparticle	Chitosan Mw: 150 kDa Degree of deacetylation: 95% Tiengene Bio-Technique Co Ltd	Polylactic acid	Periodontal bone regeneration	In vitro—bone marrow stem cells	Enhanced cell proliferation and osteogenic differentiation	117

(continued)

Table 8.1 (continued)

Scaffold type	Chitosan type*	Other ingredients	Aim	Application	Result	Refs.
Thermo responsive hydrogel	Glycol chitin (synthesized by N-acetylation of glycol chitosan)	Collagen	Odontogenic differentiation	In vitro—human dental pulp cells	Proliferation and odontogenic differentiation of human dental pulp cells	118
Solution	Chitosan Av Mw: 348 kDa Degree of deacetylation: 80%		Osteogenic differentiation	In vitro—macaque dental pulp stromal stem cells	Stimulation of cell proliferation and early osteogenic differentiation. Mineralization unaffected by chitosan treatment	119

*In some references, source and properties of chitosan was not stated; Av Mw: average molecular weight

dentistry for both drug delivery and tissue regeneration. Nevertheless, it is important to take into consideration the regulatory issues in regard to safety and quality of chitosan in development of these systems.

At present, standardization of chitosan is the most important issue in biomedical applications, and needs to be considered very cautiously. Furthermore, the choice of chitosan with appropriate properties such as source of origin, degree of deacetylation, molecular weight, etc. is very crucial in designing a therapeutic system either for drug delivery or in tissue engineering in order to achieve the desired effect. Consequently, in order to achieve a successful system, an alliance among appliers (dentists, medical doctors etc.) chitosan producers and formulators appears to be inescapable.

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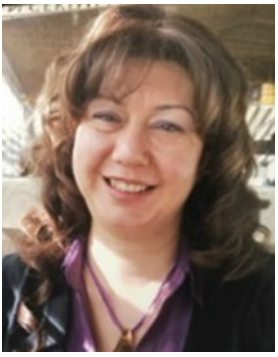

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Chapter 9
Hydroxyapatite Scaffolds Produced from Cuttlefish Bone via Hydrothermal Transformation for Application in Tissue Engineering and Drug Delivery Systems

Nefeli Lagopati and Simeon Agathopoulos

Abstract An increase in life expectancy due to improvements in healthcare, in parallel with high percentage of injures, because of traffic accidents and sport activities, has emerged as the primary reasons for the replacements of lost, infected, and damaged bones. Combined with tissue engineering, this is an area of great interest to regenerative medicine. Novel scaffolds development, providing a suitable environment that can favor osteoinduction for the newly formed bone is needed. Composite porous hydrogels, based on alginate and chitosan with the dispersed phase from powders of bioceramics, such as hydroxyapatite (HAp), are recently developed for this reason. This work presents a reverse and novel approach, where these two popular hydrogels are infiltrated in a 3D HAp-scaffold. More specifically, HAp is obtained from aragonite from cuttlefish bone via hydrothermal transformation. This reinforcement of HAp with alginate or chitosan hydrogels, through infiltration method gives to the final product proper mechanical potential for hard tissue regeneration. The structure of the produced scaffolds resembles the microstructure and the texture of the natural bone. These advanced scaffolds are easily handled by the surgeon while maintaining their porous structure during the implantation process to promote the regeneration of newly formed bone tissue. In particular, once such a scaffold is implanted in an area where the bone tissue is lost, biological liquids will be able to penetrate into the pores of the lyophilized composite scaffold. The polymeric matrix will then be dissolved and the remaining HAp, or its precursor compounds, which will eventually transform into HAp, will promote osteoinduction. The worldwide availability and the low cost of cuttlefish bone, along with their biological-natural origin are attractive features making them highly sorted material used in the preparation of advanced scaffolds containing HAp for applications in biomedicine. The optimization of the fabrication

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technique is required to unravel the endless potential of biomaterials, shedding light on this promising interdisciplinary field, which includes both tissue engineering and drug delivery system approaches.

Keywords Hydroxyapatite scaffolds · Cuttlefish bone · Hydrothermal transformation · Regenerative medicine · Tissue engineering · Drug delivery systems · Composite biomaterials

9.1 Introduction

Tissue engineering is an interdisciplinary biotechnological field which is evolved from the field of biomaterials and applies the principles of engineering and life sciences (medicine, cell and molecular biology), focusing on restoring, maintaining and finally improving tissue function or a whole organ development, when tissues or organs are severely diseased or lost due to cancer, congenital anomaly, or trauma [1–3]. This is often feasible via replacement of the damaged tissue with natural, synthetic, or semisynthetic tissue mimics, which are either be fully functional or will gradually grow into the required functionality [4]. The term usually refers to the practice of combining scaffolds, cells, and biologically active molecules into functional tissues [5]. Interaction and integration between tissues and cells through incorporation of appropriate physical and cellular signals are required. Modifying factors, such as biologically active proteins and DNA, are often utilized. Owing to the outstanding advantages, tissue engineering is often considered as an ultimately ideal medical treatment [6].

Regenerative medicine is a broad field that includes tissue engineering [7]. It also incorporates research on self-healing, recreating cells and rebuilding tissues and organs. Drug delivery systems are a distinct field that closely supports the above approaches of regenerative medicine. The process of regenerating body parts may occur in vivo or ex vivo. It requires cells, natural or artificial scaffolding materials, growth factors, gene manipulation, or combination of all the above-mentioned elements [8].

The terms “tissue engineering” and “regenerative medicine” have become largely interchangeable and sound like being scientifically alternative fields. This aspect is often generated due to the point of view of the field itself, which focuses on cures instead of treatments especially, for complex and often chronic diseases [2, 9]. Considerable advances in tissue engineering and regeneration have been accomplished over the last decades [10]. This is reasonable, considering important data related to surgical procedures, performed annually worldwide, in order to treat millions of patients, experiencing organ failure or tissue loss.

Considerable effort has been made to develop biocompatible scaffolds for tissue engineering. Actually, scaffolds matrices are utilized to fill the tissue void, to provide structural support and to deliver growth factors or stem cells, which could form tissues within the body [1]. In general, scaffolds are porous, degradable structures, fabricated

from either natural materials (collagen, fibrin) or synthetic polymers (polylactide, polyglycolide, or co-polymer of polylactide and glycolide). Their structure varies from sponge, such as sheets and fabrics, to gels or highly complex structures, with channels and intricate pores, fabricated using new materials-processing technology [4].

In order to produce engineered tissue, there are two useful approaches. Firstly, scaffolding can be used as a cell support device upon which cells are seeded in vitro, laying down a matrix for producing the foundations of a tissue for transplantation. Secondly, scaffold can be used as a growth factor/drug delivery device, meaning that the scaffold is combined with growth factors. In this way, upon implantation, cells from the body are recruited to the scaffold site and form tissue throughout the matrices. Topography, architecture and composition of scaffolds are proven to interact and influence cell behavior [11]. The use of biodegradable polymers, such as poly-lactic-*co*-glycolic acid (PLGA) for scaffolds' development, has become widespread. Some of the scaffold types include high-pressure CO_2 foamed poly-lactic acid (PLA) scaffolds, injectable scaffolds, and novel custom scaffolds [12–14].

The source of cells and the cell type in general, are also very important choices. Primary cells are often taken from the patient and used in conjunction with scaffolds to produce tissue for re-implantation. The invasive nature of this strategy makes difficulties in applications. Stem cells (embryonic stem (ES) cells), bone marrow mesenchymal stem cells (BM-MSCs), umbilical cord-derived mesenchymal stem cells (UC-MSCs) are quite promising [15].

Drug delivery is a method of administering a pharmaceutical compound to achieve a therapeutic effect in humans or animals [16]. In fact, drug delivery is an engineered technology for targeted delivery and controlled localized release of therapeutic agents [17, 18]. Nowadays, personalized medicine is the aim of biologically precise and accurate drug delivery system development, with more biological and fewer materials-oriented characteristics [19]. Tumor-targeted nanoparticle delivery system is also a challenge and has been extensively studied in tumor therapy and diagnosis [20]. Scaffolds can be used to deliver growth factors to the sites of repair, thus expediting the recovery process [21].

The clinical use of autologous and allograft bone has been proven popular in the reconstruction of bone defects [22, 23]. However, the use of autologous bone is proven to create a secondary trauma, and the allograft bone induces immune repulsion [24]. Bone grafts are avascular and dependent on diffusion [25]. The use of autologous and allograft bones is not effective in large defects, because in these cases, the allografts were resorbed by the body before the completion of osteogenesis [26]. Artificial bone-like materials, such as bone cements, bioglass and hydroxyapatite (HAp), can solve the problem [27]. The ideal scaffold, as a biomaterial, should exhibit biocompatibility without causing an inflammatory response [28]. Aragonite ($CaCO_3$), plaster of Paris ($CaSO_4{\cdot}2H_2O$), β-whitlockite ($Ca_3(PO_4)_2$), tri-calcium phosphate (β-TCP) are popular absorbable, inorganic materials that have been largely investigated for bone tissue engineering [29, 30]. Calcium phosphate ceramics (CaPs), such as TCP, HAp ($Ca_{10}(PO_4)_6(OH)_2$), and tetracalcium phosphate are also widely used, because these biomaterials are protein-free, minimal immunologic as well as they display

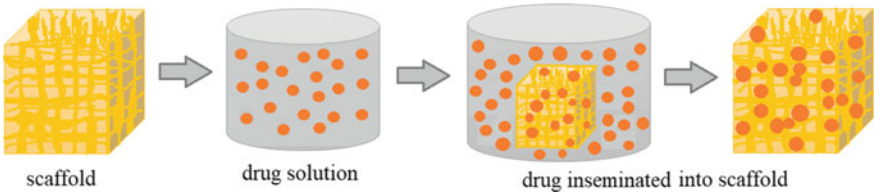

Fig. 9.1 Schematic representation of the immersion of a scaffold in a drug solution

high biocompatibility [12, 31, 32]. Generally, three-dimensional (3D) bone bioactive scaffolds used for bone repair should be based on biomaterials with adequate properties, such as biocompatibility, bioactivity, osteoconduction, osteoinduction and biodegradation [33–35]. The main method, which takes place with immersion of the scaffold in drug solution, is depicted in Fig. 9.1.

Artificial implants of HAp are very popular for hard tissue replacements, since this material allows the acceleration of bone growth around the implant [36, 37]. Biological apatites attract special interest and this is coming from the general belief that the components of HAp, namely Ca^{2+}, PO_4^{3-}, and OH^-, are regulators of the physiological osseointegration process [38]. Nonetheless, pure HAp lacks optimized mechanical properties. Thus, development of modified (or composite) biomaterials, based on HAp was a challenge.

Pacific Ocean offered the raw material, derived from corals since 1970s [39]. Scientists had immediately understood that the resultant materials had similar microstructure to the inorganic mineralized structure of natural bones. Important efforts have been made in order to develop highly bioactive bone-scaffolds of HAp from cuttlefish bone via hydrothermal transformation [40]. The advantages of this attempt include the worldwide availability and low cost of this natural material, along with the simple and inexpensive equipment needed for the whole processing. The optimal physical characteristics of HAp produced from cuttlefish bone, such as pore size, and its bioactivity and biocompatibility with osteoblasts as well as easy shaping are some other benefits of this choice [36].

Hydrothermal transformation (HT) of aragonitic ($CaCO_3$) corals is described by the chemical equation:

$$10CaCO_3 + 6(NH_4)_2HPO_4 + 2H_2O \rightarrow Ca_{10}(PO_4)_6(OH)_2 + 6(NH_4)_2CO_3 + 4H_2CO_3 \quad (9.1)$$

Cuttlefish bones have similar chemistry and crystallography as corals and could be the sea species of choice, as they are available worldwide. Aragonite from cuttlefish bones can also be hydrothermally transformed into HAp [36].

The following sections highlight the desirable characteristics of cuttlefish bone, as a source of aragonite, in order to transform it into HAp. HAp fabrication and characterization are dully clarified in the following part. Of course, the importance of this material, from a biological point of view, will be underlined, via analysis of its biochemical potential. In another section, composites with natural biopolymers will

be discussed due to the great appeal of this field the last decades. In vivo performance of these advanced materials, upon transplantation in small animals and also drug delivery systems development, based on HAp are also reported in this chapter. Finally, recent achievements as well as future perspectives will be summed up in the final section, shedding light on this promising interdisciplinary field.

9.2 Cuttlefish Bone

Cuttlefish bones or cuttlefish shells (considering that the bones of the mammals are made of Ca-salts of phosphates, whereas the shells are made of Ca-salts of carbonates) are often obtained by the species *Sepia officinalis* [41]. It can be generally accepted that cuttlefish bones have similar physical properties and configuration as corals. However, the inorganic part of cuttlefish bone is an anisotropically mineralized porous structure of orthorhombic aragonite (Fig. 9.2), which can be transformed into HAp, via the chemical reaction as shown in Eq. (9.1) [40, 42]. Their low production cost and availability make them desirable natural material. The size of their channeling pores is normally ~80 μm in width and ~100 μm in height, which immediately allows them to host physiological activities [40]. The biomineralization of cuttlefish bones has been reported in the literature over the last three decades [43]. Cuttlefish bones can be hydrothermally transformed into AB-type carbonated hydroxyapatite completely at 200 °C (A and B refer to carbonate substitution in the sites of OH^- and PO_4^{3-}, respectively, in HAp unit cell; carbonate substitution as well as the nano-crystallites of HAp (<50 nm) are very important features of biological HAp). Particularly, the highly channeled structure and the use of fresh fished cuttlefish bones both favor the efficient diffusion of the reacting solution towards the aragonite structural units, resulting in fast kinetics.

Orthorhombic aragonite is a less-stable polymorphic modification of anhydrous calcium carbonate. It is one of the most extensively investigated biomineral in nature. In comparison with the abiotic form of aragonite, this is produced under ambient conditions and exhibits more sophisticated design with enhanced mechanical properties [44, 45]. The organic matrix of cuttlefish bone provides a suitable substrate for the nucleation and controlled growth of biominerals. It consists of diverse macromolecules, mostly peptides, polypeptides, and proteins, with a modest amount of polysaccharides, lipids and pigments [46]. These protein-rich clusters, existed in the organic matrix, enable the formation of a crystal nucleus. This biomineralization process is known as a two-step nucleation mechanism [47] and through it, aragonite nanoparticles are commonly associated into hierarchically organized submicron- and micron-sized structures [48]. Soluble organic matrix (SOM) proteins seem to play an important role in morphogenesis of the cuttlefish bone's biomineral structures on a nanoscale basis [42] and found to be mainly within biominerals, i.e. intracrystalline. Proteins of the insoluble matrix are localized around the crystal phase ("intercrystalline").

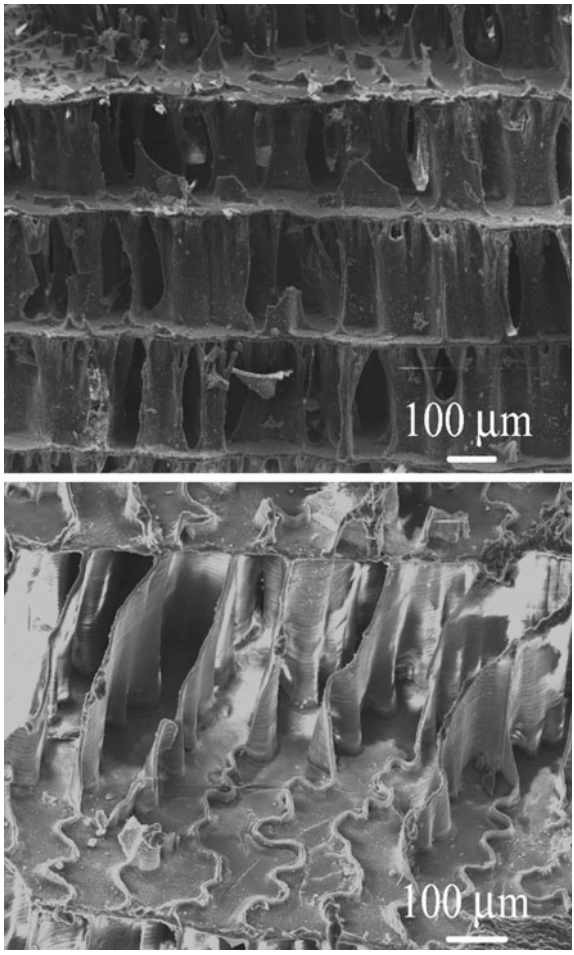

Fig. 9.2 Lamellar porous structure of HAp scaffolds and traces of the perpendicular walls among the plates. Different diffractograms (as far as the intensity of the peaks is concerned) are obtained when X-ray analysis is conducted along the planes and along the direction of the perpendicular walls

Quantitatively, polysaccharides represent the second class of important macromolecules, after proteins, in mollusk shells. In particular, they can be roughly divided in two groups: chitin and soluble acidic polymers [49]. Chitin is a long-chain insoluble polymer made of a single monomer, *N*-acetyl glucosamine. In mollusks, chitin was identified, initially, in cuttlefish bone and in squid feather and later in several other shells [50]. Chitin synthesis is catalyzed by an enzyme, chitin synthase [51]. The inhibition of the activity of this enzyme has a drastic effect on the structure. ß-type of chitin is widespread in mollusk shells. It forms with other macromolecules, especially proteins, supramolecular complexes [52].

In addition to chitin, soluble acidic polysaccharides may also be present in cuttlefish bone. Many shell polysaccharides are covalently bound to protein core, to form glycoproteins [53]. Polysaccharides are constituted of neutral, amino, and acidic monosaccharides in variable proportions [54]. Furthermore, they can be sulfated

[53]. In classical models of shell mineralization, sulfated polysaccharides play a cooperative role with proteins, by concentrating calcium ions at the vicinity of the nucleating factors [43]. Their role includes exerting additional functions, such as tissue-to-cell communication, or sequestering of water molecules.

In shells, lipids represent an extremely minor fraction of the organic matrix. Fatty acids, cholesterol, phytadienes, ketones and ceramides are normally found [55]. These lipids seem to promote the repair of the stratum corneum, the upper layer of the skin, but their role in shell mineralization is yet unknown.

Pigments are also important components of the shell, since they form patterns on the shell surface, which, in numerous cases, are species specific. Pigments are incorporated in the shells, where they seem to be bound to the shell matrix macromolecules [56]. They mostly consist of unsubstituted chains of 8–13 conjugated double bonds polyenes and carotenoids comprising unmethylated polyacetylenic backbones [57]. Pigments and some shell proteins usually form complexes.

The morphology of the cuttlefish bone differentiates from most of the biomineral structures of natural aragonite, as it is located inside the organism, in the cuttlefish bone sack. It regulates the buoyancy by using ventral chambers and provides mechanical support with its robust dorsal shield. An adult cuttlefish bone has about 100 parallel-superposed chambers, which have a complex internal arrangement of calcified pillars and organic membranes [58]. Each chamber is sealed with a hard-dorsal shield. It leaves only the caudal side open for buoyancy regulation [42]. The membranes result from a myriad of minor membranes initially filling the whole chamber, made of nanofibers evenly oriented within each membrane and are slightly rotated with respect to those of adjacent membranes, producing a helical arrangement [58]. Consequently, the chambers are composed of horizontal septa and membranes and vertical pillars and membranes [58]. The septa are divided into a chamber roof and a chamber floor. In vertical section, the floor is horizontally layered, whereas the roof is made up of vertical aragonite needles [58, 59]. The pillars extend vertically and are corrugated plates with thicknesses typically of 2–3 μm. This formation allows cuttlefish bone to withstand pressures of up to 20 atm [59]. The chambers are situated under the hard-dorsal shield, which protects its soft porous structure. The liquid which is found inside the chambers contributes to the biomineralization processes.

Chamber height is known to be influenced by aspects of the environment. Starvation causes cuttlefish to grow shorter chambers, giving them the ability of resistance to implosion [60]. Many species, such as *Sepia officinalis*, undertake seasonal depth migrations associated with growth and reproduction [61]. Particularly, in *Sepia officinalis*, more closely spaced chambers are produced in the deeper winter habitat [62]. It is very possible that cuttlefish bone secretes a chitin–protein complex. It self organizes layer-by-layer as a cholesteric liquid crystal, whereas the pillars are made by viscous fingering.

9.3 HAp Fabrication and Characterization

Hydroxyapatite (HAp) is surface-reactive bioceramic with a substantially high level of reactivity, which peaks at about ~100 days [63]. It is chemically similar to the primary mineral content of bones and teeth [64]. In literature, it is found with a Ca/P ratio of ~1.667. However, the exact stoichiometric ratio is hard to be obtained in HAp due to the different Ca/P ratios that can be adjusted, depending on the synthesis method and conditions employed [65]. Its desirable physicochemical attributes and biocompatibility make it the biomaterial of choice for hard tissues regenerative applications [66]. As it is considered as bioactive, it can support bone ingrowth and osseointegration when used in orthopedic, dental, and maxillofacial applications. HAp, as one of the most stable forms of calcium phosphate, is generally used as a coating on bioinert metallic implants [67, 68].

Bone is a dynamic tissue, rich in blood vessels, that acts as a structural and functional support in vertebrate's body. It is a natural ceramic-polymer hybrid nanocomposite. It consists of collagen (20%), calcium phosphate, e.g., HAp (69%) for bone rigidity, and water (9%). Other organic substances, e.g., proteins, polysaccharides, and lipids, are present in small amounts [69]. Collagen, located in bone tissue, has the form of fibrils with diameter approximately 100–2000 nm. HAp crystals have the shape of needles, usually being 40–60 nm long, ~20 nm wide, and 1.5–5 nm thick [70, 71]. The mineral component of bone is similar to HAp. It is a porous material that makes vascular ingrowth possible and provides oxygen and nutrients for cells [72, 73]. In addition, it contains fluoride, magnesium, sodium, and other ions as impurities. Bone is rather non-uniform in microstructure and mechanical properties. The mechanical properties of bone show strong sensitivity to porosity (5–95%), the degree of mineralization, and the orientation of the collagen fibers [66].

Different methods have been used to prepare HAp from cuttlefish bone. The most popular of these methods is hydrothermal synthesis. Time and temperature of the reaction are the main variables in this process [73–76]. Hydrothermal transformation of aragonite into HAp is proven to be effective for biomedical applications. The size of the HAp crystallites produced is in the range of 20–50 nm, which is similar to the size of bone-like apatite.

9.3.1 HAp Scaffolds Produced via Hydrothermal Transformation of Cuttlefish Bone

Cuttlefish bone is extracted from cuttlefish (*Sepia officinalis*), washed with distilled water and dried. The dorsal shield is removed, and the lamellar part is cut into small blocks of using a lancet. Differential gravimetric thermal analysis (DTA/TGA, heating rate 5 K/min, in air) is employed to evaluate the exact $CaCO_3$ content of cuttlefish bone. The samples are immersed into 5% NaClO for 48 h, in order to remove organic residues [67].

To transform cuttlefish bone into HAp, the previously mentioned hydrothermal reaction method has undergone a slight modification in recent times [77]. More specifically, an aqueous solution of 0.6 M $NH_4H_2PO_4$ is added to cuttlefish bone pieces in order to regulate the molar ratio of Ca/P = 10/6 and the pieces are placed and sealed in a polytetrafluoroethylene (Teflon)-lined stainless pressure autoclave vessel [40]. The vessel is placed in an electric furnace and heated to 200 °C (5 K/min heating and cooling rates) for 24 h (various times are tested between 1 and 24 h). The pH of the solution is 9.75 (±0.2) before sealing of the autoclave and slightly higher (9.8–10.1) after the hydrothermal transformation [40]. The integrity of the structure of the initial cuttlefish bone is perfectly maintained after the hydrothermal transformation.

The resultant HAp products are washed with distilled water and dried at 100 °C for further experiments. Then, they are fired at several temperatures between 1000 and 1400 °C with an interval of 50 K (i.e., 1000, 1050, 1100 °C, etc.) in an electric furnace (5 K/min heating and cooling rates, 1 h soaking at firing temperature).

9.3.2 Characterization of HAp Scaffolds

To evaluate the crystallographic regime of the raw and the hydrothermally converted material and particularly to determine the crystalline phases of the bulk scaffolds, X-ray diffraction (XRD) analysis is performed. XRD patterns are collected from 20° to 60° with a 2θ step of 0.02 °/s scanning rate (copper K_a radiation (λ = 1.5406 nm) is produced at 30 kV and 25 mA) [78]. Comparison between the experimental XRD patterns and the standards complied by the Joint Committee on Powder Diffraction Standards (JCPDS) and International Centre for Diffraction Data (ICDD), allows the identification of phases, using the appropriate cards for aragonite, HAp and whitlockite (TCP) [40, 41].

The average crystallite size (D) of the HAp formed is calculated using the Scherrer formula:

$$D = \frac{K_\lambda}{B_{\frac{1}{2}}.cos\theta} \tag{9.2}$$

where K is the broadening constant varying with crystal habit and chosen as 0.9 for elongated apatite crystallites [77], λ is the wavelength of Cu K_a radiation (0.15406 nm), $B_{1/2}$ is the full width half maximum (fwhm) of the 002 plane, and θ is the corresponding diffraction angle.

Other structural and chemical features of the produced HAp (such as the carbonate substitution, i.e. A and B type HA) are assessed by Fourier Transform Infra-Red transmission spectroscopy (FT-IR), in the range 400–4000 cm^{-1}, using fine powdered HAp samples in KBr pellets [40, 78]. The microstructure of the produced scaffolds is observed by field emission scanning electron microscopy (FE-SEM) under secondary electron mode, using carbon-coated samples [79].

To measure the porosity, the liquid displacement method is employed in which absolute ethanol is used as the liquid medium [80, 81]. Before the experiment, the samples, pycnometer, and liquid medium are pre-warmed to 25 °C. Porosity is calculated using the following equation:

$$Porosity(\%) = \frac{W_p - W_s}{W_p - W_e} \cdot 100 \tag{9.3}$$

where W_s is the weight of scaffold, W_p is the weight of the scaffold with ethanol in the pores, and W_e is the weight of scaffold suspended in ethanol [73].

9.3.3 Fluorine-Substituted HAp

Fluorapatite has been considered for orthopedic implants [82]. Fluorapatite is less soluble than HAp [83] and enhances remineralization. In vivo studies have shown that fluorapatite coatings feature a similar degree of bone apposition to HAp coatings but less dissolution after the first 3 months of implantation [83, 84]. HAp and fluorapatite coexist in solid solution in the naturally derived apatite from bone, which contains ~0.03 wt% fluorine [85]. Fluorine-substituted HAp has been proposed for producing implant materials for bone and dental restorations [86]. Accordingly, fluorine-substituted HAp can be considered as a bioceramic suitable for long-term implant fixation.

In order to prepare fluorine-substituted HAp, the hydrothermal transformation, previously mentioned, is boosted with some extra experimental steps. More specifically, after the addition of $(NH_4)_2HPO_4$, fluorine is introduced via NH_4F solution at two different concentrations, to achieve different levels of substitution. The other procedure is the same as reported above [36].

9.3.4 HAp Osteoinduction

Calcium phosphate ceramics are able to induce bone formation in non-bony sites, but the reasons why they can make osteoinduction, are not fully understood. This holds the key not only to the manufacture of better materials for bone repair and regeneration but also to prevention of pathological ectopic calcifications [87, 88]. Osteoinduction initially depends on the direct effect of the material [88]. Pore size and channel diameter determine cell concentration within a scaffold as well as the diffusion and availability of nutrients and oxygen. They provide the space available for bone formation and accompanying development of blood vessels. Osteoinduction also depends on the effect of the composition and solubility of the material and this phenomenon is associated with the induction of chemotaxis of stem cells and promotion of differentiation, by released calcium and phosphate ions [88, 89].

Apparently, the geometric 3D features of the HAp scaffolds from cuttlefish bone, in conjunction with their great chemical similarity to the natural bone, as described in the previous section, are important characteristics which are needed for favoring the aforementioned osteoinduction activity.

The inflammatory response to the material is also related to osteoinduction. Inflammation results in chemotaxis of osteoclast precursors that fuse to form osteoclasts and resorb the material, releasing calcium and phosphate ions. Tendency to precipitate in the presence of suitable nucleation sites and lack of inhibitors is increased by the high local concentration of ions [90].

9.3.5 HAp Biodegradation

Contemporary investigations support that there is a correlation between the grain sizes and sintering temperatures and degradation and resorption rates [91, 92]. In vitro biodegradation studies of calcium phosphates have shown that tricalcium phosphates (TCP) dissolve more rapidly than HAp [93, 94]. In vitro solubility tests of HAp, tetracalcium phosphate, and tricalcium phosphate particles have been conducted in lactate, citrate and in distilled water [95]. The results show that the solubility in distilled water decreased in the order of tetracalcium phosphate, TCP, and HAp. In lactate and citrate buffers, the solubility of tetracalcium phosphate is equal to that of TCP but still exceeds that of HAp [95].

The physiologic environment seems to significantly affect the biodegradation of calcium phosphate ceramics and that in a given site, the chemical and crystal composition, microstructure, and porosity all affect dissolution rates [94]. Generally, mechanically stable HAp implants undergo minimal dissolution [96].

A thorough understanding of the factors influencing the bioresorption of calcium phosphate implant materials has yet to be achieved. Both chemistry and material structure (especially the nano-structure) seem to contribute to dissolution. High-density implants of crystalline HAp have a low bioresorbtion rate due to their chemical composition and small surface area. Implants made of dense tricalcium phosphate exhibit a measurable dissolution rate greater than that of dense HAp [97]. Porous tricalcium phosphate implants resorb faster than porous HAp implants. Macroporosity is proven to increase the degree of dissolution and, combined with the presence of microporosity, may promote the bioresorption of all calcium phosphate materials. Finally, dissolution is affected by the environment (pH), and resorption may be controlled by cellular activity [97].

The natural origin of HAp-scaffolds obtained from cuttlefish bone, the great chemical and crystallographic resemblance between them and biological HAp as well as the possibility to regulate the ratio between HAp and β-TCP in the final products of the hydrothermal transformation of cuttlefish bone (i.e. the possibility to produce a HAp/β-TCP biphasic bioceramic scaffold) [40], can provide great opportunities for adjusting the dissolution rate of the produced scaffold according to the needs.

9.4 In Vitro Biocompatibility with Cell Cultures

In vitro biocompatibility tests of the produced scaffolds with osteoblasts have been carried out previously. Osteoblasts are cultured, for this reason, in the appropriate media supplemented with 10% FBS and 1% antibiotic-antimycotic. [3(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide] MTT colorimetric assay is employed to examine the cytotoxicity [98, 99].

Osteoblasts primary culture is prepared, since osteoblasts are isolated from the calvaria of 1–5 days old neonatal Wistar rats [100]. More specifically, calvaria have been dissected and freed from soft tissue, cut into small pieces and rinsed in phosphate-buffered saline without calcium and magnesium. These pieces are incubated with 1% trypsin-EDTA for 5 min. Four sequential digestions with 2% collagenase at 37 °C for 45 min, each, are followed, so as to produce a suspension of cells with high proportion of osteoblasts. Centrifugation at 1000*g* for 5 min is undergone, pellet is resuspended in 5 ml of fresh medium supplemented with 10% FBS and 1% antibiotic–antimycotic. The cells are finally seeded into 25 ml tissue culture flasks and led to grow in a controlled 5% CO_2, 95% humidified incubator at 37 °C [77].

The HAp-scaffolds are cut into small discs and sterilized. Osteoblasts are platted for 2 h in order to adhere to the culture plate. Then the scaffolds are gently deposited over the cells. This method avoids the cells from being carried into the pores because of capillarity action [77].

Osteoblasts' viability is evaluated by MTT assay, based on the reduction of tetrazolium salt to formazan crystals by dehydrogenase present in living cell mitochondria [98, 99]. The formazan salt formation is directly related to the amount of dehydrogenase, providing an indirect measurement of cell proliferation [101]. MTT (5 mg/mL) is added to each well. Two hours later, the cells' morphology is analyzed by inverted optical microscopy and formazan salts were solubilized with 100 μl SDS 10% HCl. Optical density is measured at 595 nm [102].

The alkaline phosphatase production (ALP) is also evaluated by the BCIP–NBT assay, based on a chromogenic reaction initiated by the cleavage of the phosphate group of BCIP by alkaline phosphatase present in the cells, ending in the production of a proton, which reduces NBT to an insoluble purple precipitate [77]. For this reason, the supernatant of each well is removed, and the cell layer was rinsed twice with PBS. Then, 200 μl of BCIP–NBT solution are added to each well. After 2 h of incubation, the cells are observed by optical microscopy and the insoluble purple precipitates are solubilized with 210 μl of SDS 10% HCl and incubated for 18 h. The optical density is then measured at 595 nm [103].

A number of studies utilizes other cell lines, such as MG63 cells (human osteosarcoma cells line), to examine the biocompatibility of HAp, prepared by cuttlefish bone, via hydrothermal method [74, 104]. In order to examine the morphology of cell population, characterization by Confocal Laser Scanning Microscopy (CLSM) is employed. For this reason, after 1, 3 and 7 days of culture, the cells are fixed and permeabilized with 4% paraformaldehyde solution in PBS with 0.2% Triton X-100. Then, they are labeled with rhodamine phalloidin for the visualization of filamentous

actin (F-actin) and 4′,6-Diamidino-2-Phenylindole and Dilactate (DAPI) to stain the nuclei. The morphology of the cells adhered to the substrate is observed by confocal laser microscopy [104].

Cell proliferation is evaluated by DNA quantification. Cell metabolic activity can also be determined with Alamar Blue Cell Viability assay, instead of MTT assay. This method is based on the fluorescence signal, measured by each sample [105].

Osteoblasts in the presence of the HAp scaffolds, produced after 24 hydrothermal transformation of cuttlefish bone confirmed their high biocompatibility. Under light microscopy observation, osteoblasts showed no evidence of morphological changes after 24, 48, and 72 h of incubation in the presence of the scaffolds, when compared to those of the control sample.

MTT data showed a pronounced increase of viability/proliferation of osteoblasts in the presence of the scaffolds, when compared to those of the control, while the production of alkaline phosphatase was not altered [77].

9.5 HAp-Composite Scaffolds with Natural Biodegradable Hydrogels

Composite materials consist of two or more materials in order to enhance some properties, usually mechanical, which cannot be achieved otherwise. For instance, advanced ceramics, reinforced with molecules, mono-crystallized fibers, zircons, or nano-molecules are designed to increase the mechanical reliability and to amplify the resistance to decay. Moreover, in bioceramics, complex biomaterials with a polymeric matrix are of crucial importance, due to their potential, such as increase tendency for osteoinduction and controlled biodegradation [106].

The HAp scaffolds produced after hydrothermal transformation of cuttlefish bone aragonite to HAp are still fragile ceramic materials. Infiltration of polymeric foamy material into this HAp scaffolds protects them from cracks, which would eventually lead to collapse [107]. The main factor affecting the mechanical properties and structural integrity of these scaffolds is their porosity.

Composite alginate-HAp scaffolds are synthesized via chelating method. Hybridic chitosan-alginic scaffolds can be also produced, which present improved mechanical tolerance and structural stability, promoting osteoinduction and osteoconduction [108].

Bioactivity of HAp is not affected if the chosen polymeric foam is biodegradable. Many scientific studies focus on synthesis of lyophilized foams based on hydrogels of alginate or chitosan. Hydrogels are very useful and handy biomaterials for applications in tissue engineering, as they can be utilized as drug or biomolecules carriers. The in vivo injection needs semi-conventional methods. Their biomimetic potential, owing to the characteristics of extracellular matrix, makes them materials with high scientific interest. High water content and flexibility are among their advantages. Their biocompatibility has been also thoroughly examined [109].

9.5.1 Reinforcement with Alginate Hydrogel

Alginate hydrogels are widely used in food, pharmaceuticals and cosmetics industry as well as in tissue engineering. Alginic acid is a natural polysaccharide, extracted by brown algae. The chemical structure of it is depicted in Fig. 9.3. Synthesis of alginate hydrogels is achievable via the dissolution/diffusion method or the internal chelating method [108, 110].

Alginate is used in wound dressings, due to its high biocompatibility and respectively low toxicity [109]. There are many applications, focusing on immobilization of cells via alginate, such as insulin adjustment in diabetes, renal failure treatment, myocardial infarction, bone deficits treatment [111].

However, the use of alginate scaffolds in tissue-engineering applications is restricted, owing to their weak mechanical properties, lack of cellular interactions and uncontrollable degradation [112]. Scaffolds made from alginate are soft and weak, constraining their further application as templates for tissue generation. Alginate scaffolds have limited protein adsorption capacity, as most cells do not adhere to the scaffold. Moreover, when the scaffold is dissolved in the medium, the loss of divalent cations into the surrounding medium causes uncontrollable degradation of alginate. To improve these limitations, the use of HAp as a reinforcing material is proposed to make novel porous alginate/HAp composite scaffolds.

Based on the typical protocol used to prepare a pure alginate sponge, alginate powder is dissolved and mixed in double-distilled water. Then the crosslinker solution is added to the alginate solution and stirred continuously with the homogenizer at 26,000 rpm for 3 min. The concentration of alginate solution is kept at a constant 3% (w/v) for all the scaffolds [113]. The alginate gel is left for 30 min before quenching and then is frozen for 6 h and lyophilized overnight with the use of a freeze dryer at a freeze-drying temperature of −45 °C.

For the preparation of composite alginate/HAp scaffolds, the desired amount of HAp powder is dispersed completely in double-distilled water with the homogenizer at 26,000 rpm for 3 min. Then, alginate powder is homogenized thoroughly with the dispersed HAp solution at 26,000 rpm for 3 min. Afterwards, crosslinker solution

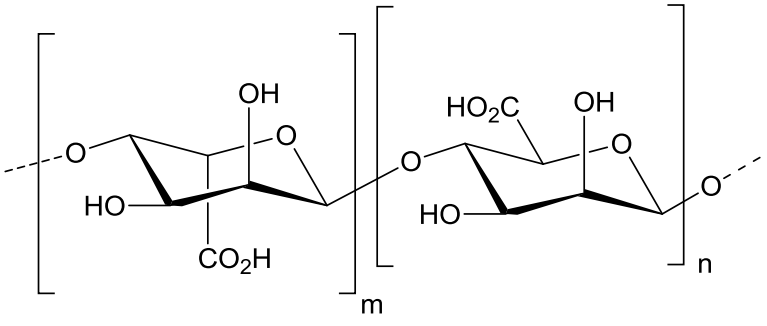

Fig. 9.3 Chemical structure of alginic acid

is added to the alginate/HAp solution and mixed completely with the homogenizer. The alginate/HAp mixture is then molded, frozen, and dried by the same procedure described above [113].

An in-situ method, which resembles the approach described in the next section for chitosan, has been attempted to produce alginate/HAp scaffolds [114]. According to this approach, Ca^{2+} cations, derived from the dissolution of calcium phosphates (HAp and β-TCP) produced from the HAp-scaffolds (from cuttlefish bone hydrothermal transformation), can trigger chelating reaction of alginate; thus, in-situ formation of alginate/HAp scaffolds can spontaneously take place.

Another method includes the production of HAp scaffold and the preparation of alginate hydrogel separately [115]. For the preparation of alginate hydrogel, alginate Na-salt is dissolved in distilled water (in a concentration of 1–2%), under continuous stirring. A second solution is prepared by dissolving calcium chloride ($CaCl_2$) in distilled water. This solution also contains a small amount of gluconolactone (GDL), which is needed to provide an acidic environment (pH ~ 4–5) to the chelation reaction. Afterwards, the second solution is added dropwise to the first solution. Then, the alginate gel is infiltrated inside the pores of the HAp-scaffold. Nevertheless, the HAp scaffold is never submerged into the alginate hydrogel as soon as it is exposed to the solution. This is due to the fact that the hydrogel is highly viscous, which makes infiltration into the scaffold almost impossible. However, the application of primary vacuum (by merely using a water-flow vacuum pump) favors the infiltration of the alginate into the porous HAp-scaffold; given that HAp is a hydrophilic material with a density of approximately 3.2 g/cm^3. Complete infiltration and submersion of the scaffold into the hydrogel is achieved by boiling the solution containing the scaffold. Heating the solution causes air bubbles to be released from the scaffold due to a decrease in pressure. The porosity of the HAp-scaffolds prepared from cuttlefish bone using hydrothermal transformation allows the penetration of alginate gel into the pores.

This resultant composite scaffold can be used as prepared (via this method), or after lyophilizing of the produced composite. Moreover, alginate can be doped with a growth factor before infiltration, which can be released gradually after the implantation.

9.5.2 Reinforcement with Chitosan Hydrogel

Chitosan (poly-(D)glucosamine) is a natural biodegradable and biocompatible polysaccharide derived by deacetylation of chitin. The structure of chitosan is depicted in Fig. 9.4. Chitosan is the only natural positively charged polysaccharide. Chitosan is insoluble in aqueous solutions (pH > 7) and is dissolved in acidic solutions (pH < 6).

Chitosan hydrogels are created with the mixture of chitosan with water-soluble polymers. The positively charged chitosan interacts with other negatively charged molecules, forming hydrogels. Proteins, such as collagen, gelatin, anionic polysac-

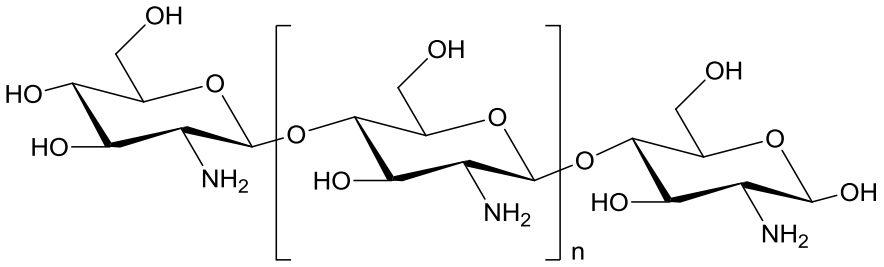

Fig. 9.4 Chemical structure of chitosan

charides, such as hyaluronic acid and alginic acid can also develop hydrogels in combination with chitosan [116].

Chitosan possesses antibacterial and antifungal properties, analgetic effect, and hemostatic activity. It can interact with cell surface molecules, which can change their permeability. Biodegradation of chitosan is significant. Due to its properties, chitosan is widely utilized in biomedical and pharmaceutical applications. Surgical sutures, dental implants, artificial skin, bone reconstruction and medication are among the most popular applications [116].

It has been shown that composite biomaterials of HAp and chitosan display increased osteoconductivity and biodegradation together with sufficient mechanical strength for orthopedic applications. In recent years, the interest in biomaterials such as HAp and chitosan has been increased significantly, evidenced by the significant growth in the number of scientific articles reporting their characterization and evaluation [117–119].

The chitosan/HAp composite scaffolds can be prepared by two different methods and a subsequent freeze-drying process to obtain an adequate morphology and pore size. Production of scaffolds of chitosan/HAp in-situ is the first method, which is mentioned. A solution of chitosan is prepared (2% w/v) by dissolving chitosan powder in acetic acid solution (1% v/v). A solution of $Ca(NO_3)_2$ 0.5 M is added in the chitosan solution under constant stirring, and finally a solution of $NH_4H_2PO_4$ 0.3 M is added drop-wise, as precursors to prepare the HAp in-situ, keeping the Ca/P ratio equal to 1.67. The total solution is mixed thoroughly for 2 h until fully homogenized, and finally the solution is put into containers and frozen for 8 h at −80 °C and lyophilized for 24 h [119].

Production of scaffolds of chitosan/HAp in powder form is the second method, which is reported. A solution of chitosan (2% w/v) is prepared by dissolving chitosan powder in a solution of acetic acid at 1% (v/v), as the first protocol. HAp powder is dispersed in deionized water for 2 h. Subsequently, the suspension is added dropwise to the chitosan solution, while the solution is being agitated. Afterwards, the chitosan/HAp suspension is vigorously mixed using a magnetic stirrer for 2 h to obtain a homogenous mixture, and then is transferred to containers, frozen at −80 °C for 8 h and lyophilized for 24 h [119].

The following method is used in the production of HAp scaffold and chitosan hydrogel individually [120]. For the preparation of chitosan hydrogel, 0.05 ml of acetic acid is added to 5 ml of distilled water. 0.1 g of chitosan is added under continuous stirring until the dissolution of chitosan. Then, the HAp-scaffold produced from the hydrothermal transformation of cuttlefish bone, is immersed in this chitosan hydrogel. Infiltration of the gel inside the pores of the scaffold takes places. The whole process and the features of the produced composite scaffold are similar to those described previously for alginate hydrogel.

9.6 In Vivo Performance

In vitro experimental data have shown that HAp scaffolds, obtained by cuttlefish bone, display good biocompatibility in addition to high bioactivity promoting osteoinduction. Therefore, a series of in vivo experiments have been conducted to establish protocols necessary for their applications in spinal cord fusion or to fill bone mineral deficits. Usually, HAp scaffolds are proven to be friable; hence, it is absolutely perceived that the microstructure of scaffolds could be easily damaged by the surgeon during implantation processes [115, 120]. To avoid this unfavorable situation, infiltration of biodegradable hydrogel into the scaffold pores have been investigated. Alginic acid hydrogel and chitosan hydrogels were chosen for this application. After hydrogel infusion, as described in the previous sections, the final composite is lyophilized and then implanted. Cracking in the scaffold will result if there is too much pressure placed on it by the surgeon during implantation. The hydrogel, after being infused into the scaffold, will act as a bonding agent or glue holding the walls of the scaffolds in place, so that its microstructure will not be damaged or destroyed. The biopolymeric hydrogel will gradually be biodegraded once a complete integration between the scaffold and the host tissue is achieved [115, 120].

Sterilization of scaffolds is carried out before the implantation using UV radiation. Then, scaffolds are immersed for 2 h in blood plasma, intended to promote the adsorption of growth factors, which are very important for the osteoinduction [120].

Wistar type rats are used in the experimental procedure. Their weight is approximately 250 g and their age about of 4–5 weeks. Their participation in the research is in compliance with all the regional, national, and international legislations. Anesthetic and analgetic substances are used to restrict the amount of pain suffered by the rats throughout the testing period. The stability and biodegradation of scaffolds are examined once they are implanted in the spines of rats. The biological effect of the scaffold on the bridging of adjacent transverse sphincters of the spine is also examined [115, 120].

The sacrifice of the experimental animals is carried out at 5, 10, 15, 20, 25, 30, 35 and 40 days after implantation and then the remaining scaffold is obtained for further examination. Scaffolds that remained at the end of week 4 after subcutaneous implantation were retrieved. There are some promising results in spinal cord fusion protocol. During observations, it was revealed that bone delicits in the spine of rats

were filled by the scaffold at the end of 4 weeks. However, there was no evidence to suggest osteoinduction has taken place. Furthermore, alginate/HAp scaffold is totally absorbed within the bodies of the rats [115]. However, it should be mentioned that when alginate hydrogel is used for any in vivo testing, partial oxidation of the polymeric chain using $NaIO_4$ must take place during the production of the hydrogel [121]. This oxidation reaction alters the structure of alginate to become a totally biodegradable compound, which can be completely passed out of the body via the kidneys.

9.7 Drug Delivery Systems

Chronic osteomyelitis is a devastating orthopaedic problem that can be treated through medical and surgical interventions [122]. Prolonged administration of systemic antibiotics is usually used [123] with serious side-effects [124]. Localized antibiotic treatment is developed to minimize such systemic complications. There are many biomaterials, which would be used as carriers of antibiotics in drug delivery systems. A number of them are non-biodegradable, such as polymethylmathacrylate (PMMA). Others are biodegradable, such as HAp, plaster of Paris, and chitosan and have been explored to replace PMMA.

There are numerous studies focusing on the development of drug delivery systems which allow drug elution to be sufficiently prolonged. An interesting attempt includes the development of a new biodegradable composite, which is composed of HAp, plaster of Paris, and chitosan. The composite slowly releases the antimicrobial drug for more than one month. In addition, the lower setting temperature of 30 °C enables impregnation with heat-labile antibiotics [125, 126]. The composite is impregnated with antibiotics and then tested in osteoblasts, for cytotoxicity and biocompatibility MTT tests.

A wide variety of applicable drugs as well as functional groups frequently used to bind drugs to the calcium phosphate substrate opens up an almost unlimited number of variables in technologies used for the preparation of HAp/drug compositions intended for targeted and controlled drug delivery [127].

Small (<10 μm) and interconnected pores provide the appropriate conditions needed for the preparation of an effective drug delivery system. These pores could carry pharmaceutical substances and release them relatively slowly. The size, amount, and interconnectivity of pores are mainly responsible for the drug release [128, 129]. Lower porosity of the bioceramic scaffold gives a larger initial burst release of drugs [130]. Many studies demonstrate that pore sizes need to be in accordance with the size of drug molecules or other bioactive molecules in order to prepare a slow-release drug delivery system [131]. Actually, it is shown that interconnected pores with an average diameter of more than 100 μm are essential for bone cell ingrowth and proliferation responsible for the new bone formation.

It can be concluded that ceramic/polymer scaffold preparation for drug delivery system applications can be performed using three strategies that are mentioned below.

The first strategy proposes that drug can be incorporated into a HAp scaffold prior to the deposition of a polymeric coating. The second method suggests that drug can be incorporated within the polymer coating material. Indeed, growth hormone has been successfully conjugated (by using carbodiimide conjugate chemistry) in alginate hydrogel [132], and the release of the hormone, both in vitro (at 37 °C) and in vivo (in myocardium of rats), lasted for about 2 weeks [133, 134]. Thus, it can be plausibly assumed that the kinetics of this release will be further prolonged if the alginate hydrogel is infiltrated in the pores of the produced HAp-scaffolds, obtained from the hydrothermal transformation of cuttlefish bone. The third strategy supports the idea that drug is absorbed on HAp scaffold that is pre-coated with polymer [127].

The combination of ceramic preparation technologies is used in order to develop complex three-dimensional structures with hierarchical pore structures of HAp. Development of preparation technologies will help to obtain the artificial bioceramic structures very close to the natural bone. Complex three-dimensional porous HAp structures could be used not only for the delivery of one therapeutic agent, but also as a matrix for delivering more than one drugs at the same time [127]. Mother Nature provides this complex matrix in the shape of cuttlefish bone, which can be easily and efficiently transformed into HAp scaffold.

9.8 Concluding Remarks and Perspectives

Considerable advances in tissue engineering and regenerative medicine have been accomplished over the last decade [2]. Nowadays, basic functional tissue engineered strategies have been of crucial importance. Thus, there is still considerable scope for future development, such as cell sources, individually tailored cell supports, immune modulation as well as vascularization. The predictive abilities of computer and mathematical modelling for more complex materials are encouraging [1].

HAp is a promising biomaterial in bone regeneration. Fabrication of HAp nanobioceramics is thought to be another effective choice to enhance their mechanical properties, since the nano-sized ceramics exhibit better mechanical properties compared to conventional micro-sized ceramics [135].

Synthetic HAp bioceramics are difficult to be resorbed within the physiological environment, which has hindered their use as bone grafts, tissue engineering scaffolds, drug carriers, and other applications. Using a secondary phase with a faster degradation rate as the composite component, e.g. tricalcium phosphate, calcium carbonate, bioglass, silicate, etc., is considered a simple method to regulate the degradation rate of HAp-based materials. The rate of biodegradation increases as more additive materials are used [136–138]. HAp bioceramics possess good biocompatibility and high osteoconductivity. Traditional synthetic HAp bioceramics are still generally considered to lack sufficient bioactivity and osteoinductivity, so as to induce osteogenic differentiation of the stem cells and osteoblasts stimulating new bone formation [68].

HAp materials are widely used in hard and soft tissue repair, bone tissue engineering, drug/gene/protein delivery, chromatography, imaging, and diagnosis. Chemical compositions and structures of HAp have a great impact on its performance. Therefore, there are still many challenges that need to be further investigated in detail [139]. First of all, as the physical, chemical, and biological properties of HAp are very important criteria, a strategy to control the chemical composition and structure of HAp has to be developed to meet the specific requirements for each particular case, because the real mechanisms for controlling these properties need to be comprehensively researched and confirmed. Secondly, as a drawback of HAp materials is their low degradation rate, controllable degradation rate is a challenge [68]. In this way, applications as bone grafts, bone tissue engineering scaffolds and degradable drug delivery materials will be achievable [68].

As far as drug delivery system development is concerned, another important issue is that it is still difficult to synthesize uniform HAp particles with mono-dispersion and narrow size distribution in a large scale [139]. This is very important for application of HAp as drug carriers. Additionally, the development of the HAp-based nanoparticles with diagnostic and therapeutic potential is another challenge for future HAp applications [68].

Last but not least, the fabrication of HAp materials with excellent osteoinductive properties is an important future perspective, due to the potential applications for regeneration in large-sized bone defect or bone reconstruction using bone tissue engineering technology. Nano-/macro-structured surface design will be the most effective and low-cost approaches to achieve this objective [139].

Accordingly, the above statements clearly suggest that the field of bone reconstruction and regeneration, by means of tissue engineering and regenerative medicine, is still open and full of challenges, where HAp-scaffolds occur directly from Mother Nature, like cuttlefish bones hydrothermally transformed, can play an important role.

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Chapter 10
Marine Nanopharmaceuticals for Drug Delivery and Targeting

Innocent J. Macha, Besim Ben-Nissan, Wolfgang H. Müller and Sophie Cazalbou

Abstract The current need for new medicines with reduced toxicity, enhanced bioavailability as well as improved drug efficacy and patient compliance is more pressing than ever before. Clinical active agents can now be reformulated with the help of nanotechnology into "nanopharmaceuticals" with superior pharmacokinetics for site-specific delivery. With the available nanotechnology, studies suggested that marine drugs hold tremendous promise to bring forth novel medicines for the treatment of a wide range of human diseases, but unfortunately this promise has yet to be fully realized. Deadliest diseases such as cancer, HIV/AIDS, and neurological disorders, just to mention few, can be halted by using marine nanopharmaceuticals, which are cost-effective natural products. Legal and scientific frameworks have to be in place with full support from global human health communities to create a unique set of opportunities in the cause of biodiscovery and marine drug development processes.

Keywords Marine nanopharmaceuticals · Anti-cancer · Anti-HIV/AIDS · Neurological disorders · Multiple sclerosis

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10.1 Introduction

Nanotechnology is defined as the study and use of materials in the size between 1 and 100 nm, where properties such as physical, chemical, biological, electrical, optical, and mechanical differ significantly from those at a larger scale. Nanotechnology is a highly multidisciplinary field, drawing from fields such as applied physics, materials science, colloidal science, device physics, supramolecular chemistry, and even mechanical and electrical engineering. The properties of materials change when their size approaches the nanoscale and when the percentage of atoms at the surface of a material becomes significant. Specifically, as a particle's size decreases, a greater proportion of its atoms are located on its surface relative to its core, often rendering the particle more chemical reactive because of an increased surface area, increased dissolution rate and improved performance.

Nanotechnology has revolutionized a wide range of field and has shown great potentials in medicine with the development of nanomedicines or nanopharmaceuticals. Nanopharmaceuticals began to appear on the market more than a decade ago, with some becoming bestsellers in their therapeutic categories. Nanopharmaceuticals have made significant clinical impacts in the treatment of cancer, Central Nervous Systems (CNS) disorders, cardiovascular disease, and infection control. It has recently been reported by BCC Research that the global nanomedical market was valued at $134.4 billion in 2016 and is projected to grow at a compound annual growth rate (CAGR) of 14.0% from 2017 to 2020, and should reach $293.1 billion by 2022 from $151.9 billion in 2017 [1].

Pharmaceutical industries though undergoing revolution, still face enormous challenges, ranging from revenue losses due to patent expirations on blockbusters to greater regulatory oversight and ever-increasing challenges from generic drugs manufacturers. Due to high cost (>$800 M) and time involved (10–15 years) in developing and launching a new drug to the market pharmaceutical industries are more focused on shareholder profits than innovative therapies. It has been speculated that nanopharmaceuticals may be the answer and pharmaceutical industries are turning to nanotechnology to enhance or supplement drug target discovery and drug formulation [2] as a response to the socio-economic changes that are occurring in the world. Nanopharmaceuticals provide an alternative method to drug delivery using nanosized structures which have less side effects, quicker drug infusion, improved drug bioavailability, more efficient treatment and can be approved by FDA as de facto "new drug" for marketing purposes. It opened up a new approach for developing new drugs at a fraction of the cost of more traditional development.

Nanopharmaceuticals entail the use of numerous materials and potential pathways of different nanosized drug delivery and targeting systems that can be used to create new combinational drugs. These include nanoparticles, nanoemulsions, liposomes, dendrimers, nanocapsules, lipid nanospheres, nanotubes, colloidal particles, and quantum dots, to name a few. In this chapter we will focus on marine nanopharmaceuticals to illustrate the significant advantages they possess over the current drugs administration. The chapter will as well give an overview about presently approved

nanopharmaceuticals of marine origin and other marine products potentially for nanopharmaceuticals.

10.2 Biodiversity of Marine Environment

Oceans cover more than 70% of the Earth's surface, representing the largest ecosystem on the planet with 80% of world's plant and animal species. It is estimated that 2.2 million different kind of species are present in marine environment although 91% of them still await description [3]. According to The World Register of Marine Species (WoRMS) there are about 240,000 known species. The first census of marine life conducted for a decade and published in 2010 reveals that numerous new species have been detected with the highest number of species found in the sea around Australia (32,889) and Japan (32,777). Approximately 60% of marine animals belong to the invertebrates. It was concluded that at least 50% and potentially above 90% of marine species remain undescribed by science. Marine species range in size from the microscopic, including plankton and phytoplankton which can be as small as 0.02 μm, to huge cetaceans (whales, dolphins and porpoises) which in the case of the blue whale reach up to 33 m (109 feet) in length, being the largest known animal.

While it is true that a wide range of marine species are limited and protected, similarly there are also a variety of materials that are readily available and abundant and have yet to be exploited for their possible use. Marine organisms possess a vast range of properties, which portray a lot of their appropriate biomedical application potentials when used either directly, modified or as templates for bio mimicking. It is and will remain a humble and smart move to learn from nature and try to copy faithfully the vital components so as to develop implantable biomaterials to mimic natural tissues or organs efficiently in order to substitute diseased tissues or organs effectively, to stimulate the body's own regenerative mechanisms, and eventually to promote tissue healing [4].

The search for new drugs, drug candidates and other metabolites from marine organisms has resulted in the isolation of more than 30,000 compounds many of which are endowed with pharmacodynamics properties. It has been indicated that over 1,000 new marine compounds are discovered each year since 2008 with structural novelty, complexity and diversity [5].

10.2.1 Current Nanopharmaceuticals in Clinical Application

Nanopharmaceuticals have shown potentials to offer great benefits to patients such as improved drug bioavailability, enhanced drug apparent solubility and dissolution rate, improved dose proportionality, controlled release of drug and ability to act as immunological adjuvants in vaccine administration. It has been shown that nanopharmaceuticals can even cross the blood-brain barrier (BBB) and the blood-retina barrier

(BRB). Moreover, nanopharmaceuticals can be delivered via dermal and transdermal routes. Liposomal nanopharmaceuticals are being developed to enhance skin permeation with reduced loss of drugs due to percutaneous absorption. Lung diseases can now be effectively treated with nanopharmaceuticals through pulmonary delivery route with possible high systemic drug bioavailability and faster onset of therapeutic active [6].

Number of clinically active agents could be delivered in the form of nanopharmaceuticals through different routes. Both low water solubility and high-water solubility drugs can be re-engineered using nanotechnologies to improve their bioavailability to address the inefficient treatment and increased risk of toxicity. Hydrophobic drugs could be processed using a proprietary attrition-based wet-milling technique that reduces the drug particle size to less than a micron, which substantially increases the surface area and improves solubility. The nanoparticles are then surface treated to reduce agglomerations so they behave almost like solution. On the other hand, high water-soluble drugs could undergo the same process and then coating the nano-drug particles with relatively less soluble compounds that controls the drug solubility.

Studies have shown that re-engineering of the old drugs of that sort, improves drug bioavailability compared with the commercial products [7]. FDA has approved different nanopharmaceuticals in terms of their materials categories, polymers nanoparticles, liposomes, micelles, metallic particles, and protein-based nanoparticles. Table 10.1 shows selected nanopharmaceuticals approved by FDA since 2000.

Among all materials for nanomedicines, polymers perhaps are the most used due to their simplest form with tunable properties for specific therapeutic outcomes. It is not surprising that polymeric drugs were in the top 10 bestselling drugs in the US in 2013.

10.3 Marine Nanopharmaceuticals

The first marine product to be discovered was the dye Tyrian purple extracted from marine molluscs by the Phoenicians about 1600 BC. Marine drug development started in 1950s with the discovery of spongothymidine and apongouridine from the Caribbean sponge *Tethya crypta* [8]. Several marine based drugs have been discovered since then, from antiviral to cytostatic drugs to diagnostic tools with effective clinical use. Different techniques have been established over the years for the isolation of marine bioactive compounds from various marine organisms. It is now believed that more than 35,000 metabolites with excellent pharmacokinetics and/or intercellular delivery properties can be extracted from marine organisms. Table 10.2 shows the FDA approved nanopharmaceuticals derived from marine organisms.

There are a number of marine-derived compounds that are in different stages of drug development processes. It was estimated that over the years 1998–2006 about 592 marine compounds which demonstrated pharmacological activities, were under pre-clinical evaluation [9]. The reason why there are only few FDA approved marine based nanopharmaceuticals is the possible long-term insufficient supply of some of

Table 10.1 Selected FDA approved nanopharmaceuticals

Name	Description	Indication (s)	Approval year (s)
VISUDYNE®	Liposomal Verteporfin	Ocular histoplasmosis	2000
PEGLNTRON®	PEGylated interferon alfa-2b	Hepatitis C	2001
PEGASYS®	PEGylated interferon alfa-2b	Hepatitis B and C	2002
NEULASTA®	PEGylated filgrastim (granulocyte colony-stimulating factor)	Febrile neutropenia, in patients with nonmyeloid malignancies, prophylaxis (SC)	2002
ELIGARD®	Leuprolide acetate and polymer (PLGH (poly (DL-Lactide-co-glycolide)	Advanced prostate cancer	2002
NEULASTA®	PEGylated GCSF protein	Neutropenia, Chemotherapy induced	2002
ESTRASORB®	Micellar estradiol	Menopausal therapy (systemic delivery of estradiol)	2003
SOMAVERT®	PEGylated HGH receptor antagonist	Acromegaly	2003
OsSATURA®	Hydroxyapatite	Bone substitute (mimic bone structure allowing cell)	2003
MACUGEN®	PEGylated anti-VEGF aptamer (vascular endothelial growth factor) aptamer	Intravitreal, Neovascular, macular degeneration age-related	2004
OSTIM®	Hydroxyapatite	Bone substitute (mimic bone structure allowing cell)	2004
MIRCERA®	Methoxy PEGylated epoetin beta	Anemia associated with chronic kidney disease	2007
DOXIL®	Liposomal doxorubicin	Ovarian cancer	2008
FERIDEX®	SPION coated with dextran	Imaging agent	2008
CIMZIA®	PEGylated antibody fragment of a humanized antibody	Crohn's disease, rheumatoid arthritis, psoriatic arthritis and ankylosing spondylitis.	2008

(continued)

Table 10.1 (continued)

Name	Description	Indication (s)	Approval year (s)
FERAHEME®	Ferumoxytol SPION with polyglucose sorbitol carboxymethyl-ether	Iron deficiency anemia in adults with chronic kidney disease	2009
NANOTHERM®	Iron oxide	Glioblastoma	2010
KRYSTEXXA®	PEGYlated porcine-like uricase (Polymer-protein conjugate)	Chronic gout	2010
MARQIBO®	Liposomal Vincristine	Acute Lymphoblastic Leukemia	2012
PLEGRIDY®	Polymer-protein conjugate (PEGylated IFN beta-1a)	Multiple Sclerosis	2014
ADYNOVATE®	Polymer-protein conjugate (PEGylated factor VIII	Hemophilia	2015
ONIVYDE®	Liposomal Irinotecan	Pancreatic Cancer	2015

Table 10.2 List of approved marine nanopharmaceuticals

Name	Description	Indication (s)	Approval year (s)
YONDELIS®	Isolated from ascidian Ecteinascidia turbata	Advanced soft tissue sarcoma	2015
ADCETRIS®	Extracted from marine mollusk/cyanobacterium	Anaplastic large T-cell systemic malignant lymphoma	2011
HALAVEN®	Derived from a marine sponge (Lissodendoryx sp.)	Metastatic breast cancer	2010
PRIALT®	Derived from marine cone snail Conus magus	Analgesic for severe and chronic pain	2004
LOVAZA®	Ethyl esters of eicosapentaenoic acid derived from marine microalgae Nannochloropsis	Thrombocyte aggregation and antihypertensive	2004
VIRA-A®	From Caribbean sponge Tethya crypta	Antiviral (against herpes simplex and varicella zoster viruses)	1976
CYTOSAR-U®	Extracts (cytosine arabinoside) from marine sponge Tectytethya crypta	Acute myeloid leukemia (AML), Acute lymphocytic leukemia (ALL), Chronic myelogenous leukemia (CML), and non-Hodgkin's lymphoma	1969

marine organisms or compounds in regards with the preservation of marine environment. While it is true that a wide range of marine organisms are limited and protected, most of them can be grown in laboratory in an ecological and sustainable way [10]. Few years ago, scientists from the coral conservation group, Secore International, showed that lab-grown coral could reproduce when transplanted back into the ocean. Marine bacteria have great potentials in nanopharmaceuticals. Their isolation and possible laboratory culture will address the insufficient supply and is the essential prerequisite for discovery nanomedicines research. On the other hand, marine nanopharmaceuticals have shown a promising future evidencing the current seven (7) FDA approved nanopharmaceuticals, six (6) products in phase III, eight (8) in phase II and nine (9) in phase I of drug development processes.

10.3.1 Untapped Potentials from Marine Organisms

The vast majority of compounds or chemicals accumulated in marine living organisms with pharmacological activities are not yet fully exploited for clinical applications. The ocean has given us an opportunity to learn and possibly copy vital components or processes for the development of medicines to treat a wide range of human diseases. With modern technologies, there are high possibilities that we have everything we need to improve human health from conception to aging and death if we can ensure the future success of marine natural products as therapeutic outcomes. In additional, different research studies have suggested that lifesaving drugs are abundantly present in marine organisms. There are still compounds trapped in marine organisms where we believe that they present great potentials and they should not be overlooked. Naturally marine organisms produce diverse secondary metabolites including peptides, steroids, porphyrins, alkaloids, polyketides and terpenes to defend themselves against adverse conditions such as pathogens for their survival [11]. Most of these compounds do not play essential roles to their in situ life but they display strong pharmacokinetic activities.

10.3.1.1 Antitumor/Anticancer

Cancer is the deadliest disease and was reported to be the second most common cause of death after cardiovascular in Europe and USA [12]. Marine secondary metabolites from invertebrates are among sources of unexploited drugs, which believed to be potentially active for treatment of different diseases including cancer. Polyketide pederin derived marine sponge has been demonstrated to have remarkably antitumor activity. Marine sponge *Theonella Swinhoei* and Rove beetles of the genera *Paederus* and *Paederidus* possess genes that can be isolated and used for the biosynthesis of the polyketide structure with secretory properties of antitumor [13]. Other marine organisms identified as polyketide sources are Caribbean tunicate *Trididemmumsolidum* [14], sponge *Lithoplocamialithistoides* from Madagascar [15], bacteria

Salinosporatropica, from marine sediment in the Bahamas [16], and from Bahamian deep water sponge *Discodermia dissolute* [17]. 7-Phloroeckol is one of the polyphenols, secondary metabolites present in marine seaweed, brown algae *Ecklonia cava* with anticancer potential for human breast cancer [18]. Table 10.3 shows the selected marine drugs for cancer treatment in their different stages of testing, from US National Library of Medicine.

10.3.1.2 Anti-HIV/AIDS

The Human Immunodeficiency Virus (HIV) is a global epidemic disease and one of the top ten causes of death globally. It has claimed lives of 35 million people. It was estimated that about 36.7 million people were living with HIV at the end of 2016 and about 1.8 million people become newly infected in the year 2016. For years in-depth scientific understanding of HIV, its prevention strategies and treatment have been developed with the help of scientific and global health communities, government and civil society organizations but yet people who are living with HIV or are at risk for HIV infection do not have access to treatment and still there is no cure. The present treatments only control the virus and help HIV infected people to live healthy and relatively longer with low risk of transmitting the virus.

It was suggested that vaccine could be the only hope to solve HIV problem, but effective vaccine is far from realization. Moreover, some of the HIV antiretroviral therapy in the market have developed adverse side effects to patients which limit their therapeutic usefulness [19]. It is therefore crucial to develop effective and affordable HIV drugs so as to improve drug accessibility and save mankind. In the last decade more than 132 compounds were isolated from marine organisms and displayed strong anti-HIV activity with half of them from marine sponge alone [20]. Alkaloids and cyclic depsipeptides derived from marine sponge reported to demonstrated strong anti-HIV activity. Other marine derived compounds potential from HIV treatment, such as sulfated polysaccharides from marine algae. Lectins from marine invertebrates were also reported as potential for HIV treatment. Some of these compounds are in different stages of drug development processes as shown in Table 10.4. Most of the discovered marine anti-HIV drugs are yet to be screened and tested for drug development. It seems though the screening procedures and testing is not as easy as cytotoxicity or antibacterial tests. The emphasis should be focused on developing these procedures if we want to realize the benefit of what nature has offered in the treatment of HIV/AIDS.

10.3.1.3 Neurological Disorders

The potential of marine nanomedicinal products have been evident in the area of nervous systems diseases including Alzheimer disease (AD) and other dementias, neuropathic pain and other headache disorders, multiple sclerosis, Parkinson's disease, neuroinfections and brain tumours [30–32]. It has been shown that AD is the

Table 10.3 Selected marine antitumor nanopharmaceuticals

Name	Description	Function	Status
SOBLIDOTIN	Peptide derived from marine bacterium	Cancer treatment by inhibits tubulin polymerization, resulting in cell cycle arrest and induction of apoptosis.	Phase III (TZT 1027)
LURBINECTEDIN	Alkaloid derived from marine tunicate *Symplegma rubra*	Ovarian and breast cancer by inhibiting RNA Polymerase II	Phase III (PM01183)
CEMADOTIN	Peptide isolated from the mollusk *Dolabella auricularia. Or Symploca sp*	Mitosis inhibitor potentially for the treatment of solid tumors	Phase II (Discontinued)
DENINTUZUMAB MAFODOTIN	A pentapeptide originally isolated from the marine mollusk Dolabella auricularia.	Antineoplastic activity inhibits microtubule assembly, resulting in the formation of tubulin aggregates and inhibition of mitosis	Phase II (No active clinical trials)
MARIZOMIB (SALINOSPO-RAMIDE A)	Beta-lactone-gamma lactam isolated from marine Bacterium	Proteasome inhibitor that that prevents the breakdown of proteins involved in signal transduction which blocks growth and survival in cancer cells.	Phase II (NPI-0052)
BRYOSTATIN 1	Macrolide lactone extracted from marine bryozoan	For unresectable or metastatic solid tumors, stop the growth of tumor cells, either by killing the cells or by stopping them from dividing.	Phase I (Completed)

Table 10.4 Selected marine based anti-HIV compounds

Marine organisms	Compound	Function	References
Seaweed: *Dictyota mertensii, Lobophoravariegata, Spatoglossum schroederi andFucus vesiculosus*	Polysaccharide (Salfated Fucans)	Inhibit HIV RT	[21]
Brown algae: *Adenocystis utricularis*	Polysaccharide (Galactofucan fractions)	Anti-HIV	[22]
Sponge: *Mycale sp*	Polypeptide (Mycalolide B)	Inhibit the transport processes of HIV-1	[23]
Sponge: *Siliquariaspongia mirabilis*	Depsipeptide (Mirabamide A, B&C)	Inhibiting HIV-1 cell fusion	[24]
Tunicate: *Didemnum molle*	Peptide (Mollamide B)	Anti-HIV	[25]
Sponge: *Iotrochota baculifera*	Alkaloid (Baculiferin A–O)	Anti-HIV and Inhibit of HIV-1 entry: binds to gp41	[26]
Red algae: *Callophycus serratus*	Terpenoid (Inhibit of HIV-1 entry: binds to gp41)	Anti-HIV for HIV strains UG/92/029 and 96USHIPS7	[27]
Seaweed: *Thalassia testudinum*	Pplyphenol (Thalassiolin B)	Inhibit HIV integrase (IN)	[21]
Fungus: *Trichoderma viride*	Polyenyne (Rezishanone)	Inhibit HIV reverse transcriptase (RT)	[28]
Fungus: *Trichoderma sp.*	Sterol (Cholesta-7,22-diene-3b, 5a, 6b-triol)	Inhibit HIV Protease (PR)	[29]

pathological attack of the brain regions that are highly associated with mental functions, neocortex and hippocampus. This includes the deposition of beta-amyloid in senile plaques, the loss of pyramidal and synapses neuron and the formation of intracellular neurofibrillary tangles contains hyperphosphorylated protein [33]. Currently, the major therapeutic strategies are based on the cholinergic hypothesis and specifically on AChE inhibition [34]. Onchidal (1) isolated from mollusc *Onchidella binneyi*, cyclic guanidinium (2) like compound isolated from coral zoanthid *Parazoanthus axinellae* and 3-alkylpyridinium salt polymers (3) (Poly-APS) from marine sponge belonging to the order *Haplosclerida* (Fig. 10.1) have demonstrated strong ability to inhibit acetylcholinesterase (AChE) activity [35, 36] for the treatment of Alzheimer disease.

Antineurogenic inflammatory marine based compounds, Totepsin D isolated from marine sponge *Spongosorites sp,* and Manzamine (A–F) (alkaloids) isolated from marine sponges of the genus *Haliclona* demonstrated anti-inflammatory activity and inhibition of reactive oxygen species associated with Alzheimer disease. Ziconotide,

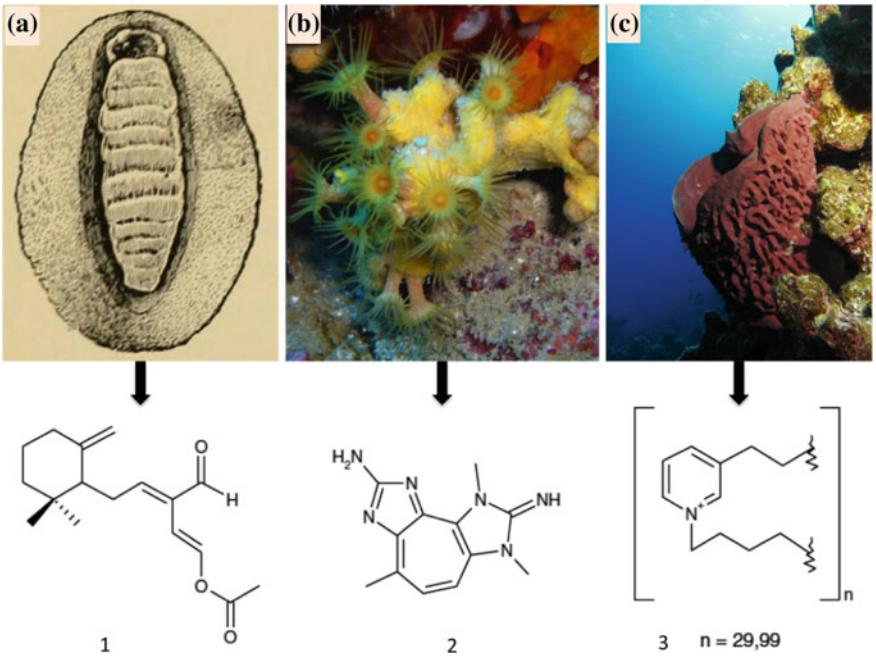

Fig. 10.1 Acetylcholinesterase inhibitors form marine organisms **a** mollusc *Onchidella binneyi* **b** coral zoanthid *Parazoanthus axinellae* **c** marine sponge *Haplosclerida*

an analgesic agent derived from *Conus magus,* a marine cone snail, it is the synthetic form of an ω-conotoxin peptide and has been approved by FDA for chronic pain associated with cancer and as an analgesic for neuropathic pain.

Multiple sclerosis (MS) is one of the neurodegenerative diseases, in which the insulating covers of the nerve cells in the brain and spinal cord are damaged and disrupts the flow of information within the brain and between the brain and body. The available therapies attempt to improve nerve functions after an attack and prevent new attacks in the early stages of the disease, but unfortunately there is no known cure. Treatment strategies to preserve tissue, promote repair and regeneration to restore the functions (neuroregenerative medicines) will provide better options for MS patients. Studies indicated that axonal injury is also among predictors of MS clinical disease. There are only few marine drugs tested for halting the progression of multiple sclerosis. The drug developed with 90% from marine king prawn *Penaeus latisculatus* suggested to be safe and effective in treating MS [31]. A research group from the University of Wollongong in New South Wales, Australia is working marine microtubule stabilizing agents on cell growth [37]. It is believed that preventing cell division by blocking the function of the cytoskeleton of the cell that is essential for separation of the chromosomes during mitosis does not only help cancer patients but also autoimmune disease multiple sclerosis patients. Hamigeran (brominated terpenes) isolated from marine micro algae, and hamigran B from marine sponge

have chemical structure similar to steroid (clinical therapy for MS) are being tested for the treatment of MS [37]. It is argued that marine based products have potential in the development of effective therapeutics for neurodegenerative disorders with excellent pharmacokinetics.

10.4 Concluding Remarks and Future Perspective

It is evident that marine derived nanopharmaceuticals play vital roles in the treatment of wide range of human diseases including non-curable diseases. There are high possibilities to design and deliver to the human body in different routes, novel multifunctional marine based pharmaceuticals because of the availability of active ingredients in marine organisms. Until now only few marine nanopharmaceuticals are in clinical use with others in different stages of drug developing processes. Efforts should be focused on the isolation of these ingredients from marine species and biosynthesize them in laboratory in a large scale or grow marine species in laboratory as the best way to address the insufficient supply challenge. Furthermore, there is a need for the screening mechanisms for marine drugs especially anti-HIV/AIDS so as to speed up the development processes. Combined efforts from different disciplines of scientists, global health communities, governments and organizations will result into quick drug development from marine organisms with unprecedented benefits for clinical applications. Moreover, it will enhance better understanding of the fundamental principles governing marine active ingredients as well as their structural organization details at the molecular and atomic level particularly in inhibiting or treating human diseases.

Apart from combining research efforts there is also a need to combine drug discovery and conservation of biodiversity and economic development at local level. The search for new drugs from marine organisms should be conducted using the best methodologies and scientific approaches focusing to commercialization of drug product. Countries surrounded by ocean should provide a fair and transparent accessibility to the marine resources ensuring reliable access to scientists.

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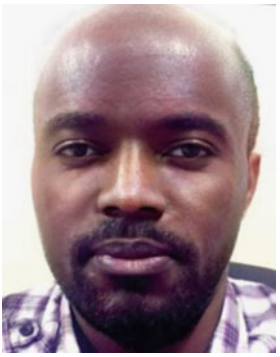

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He has successfully developed materials for implant technology such as ceramic knee prosthesis, calcium phosphate based bioactive materials, bone graft production and bio-composites,

and conducted research on biomechanics and modelling such as jaw bone, knee and hip joints, reliability and implant design modular zirconia ceramic knee prosthesis, femoral head and taper stresses and artificial ocular implants and bionic eye and recently on 3D printing of bioceramics and metallic implants and anti-microbial multifunctional coatings for drug delivery which are supported by the European Commission and the Australian Academy of Science research grants.

Since year 2000 he has published over 200 fully refereed papers in journals, and a book and 43 book chapters. He edited a book on Calcium phosphates and working on a second one on the use of Marine Structures in the Biomedical field. He is the editor of the Journal of the Australasian Ceramic Society. He was awarded by the Australian Ceramic Society's prestigious award for his contributions to the "Ceramics Research & Development and Education in Australia". For his research on multifunctional nanocoatings he also received "The Future Materials Award".

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Chapter 11
Brown Algal Polyphenol and Its Pharmaceutical Properties

Thanh Sang Vo, Dai Hung Ngo and Se-Kwon Kim

Abstract The world's oceans represent an enormous resource for the discovery of potential therapeutic agents. During the last decades, numerous novel compounds have been isolated from marine organisms and many of them have been applied for phamacological industry. Notably, marine algae are known to be one of the most important producers of variety of chemically active metabolites. Among them, phlorotannins, a polyphenol from brown algae, have been revealed to possess numerous biological activities such as UV-protective, anti-oxidant, anti-viral anti-allergic, anti-cancer, anti-inflammatory, anti-diabetes, and anti-obesity activities. Therefore, phlorotannins are considered as promising agents for the development of pharmaceuticals. This contribution focuses on phlorotannins from brown algae and presents an overview of their biological activities and health benefit effects.

Keywords Brown algae · Phlorotannins · Antioxidant · Pharmaceuticals · Biological activities

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11.1 Introduction

The marine environment represents approximately half of the global biodiversity. It is a rich source of structurally diverse and biologically active metabolites, which are important for the discovery of potential therapeutic agents [1, 2]. During the last decades, marine organisms have received much attention in screening marine natural products for their biomedical and pharmaceutical potentials [3–5]. Various marine organisms such as algae, tunicates, sponges, soft corals, bryozoans, sea slugs, mollusks, echinoderms, fishes, microorganisms, etc. have been subjected for isolation of numerous novel compounds. Consequently, numerous active agents such as lipid, protein, peptide, acid amine, neurotoxins, polysaccharides, chlorophyll, carotenoids, vitamins, minerals, and unique pigments have been discovered. Many of these substances have been demonstrated to possess interesting biological activities [6–14].

Notably, marine algae are known to be one of the most important producers of biomass in the marine environment. Algae are very simple chlorophyll-containing organisms composed of one cell or grouped together in colonies or as organisms with many cells [15]. Therefore, they vary greatly in size from unicellular of 3 to 10 μm to giant kelps up to 70 m long [16]. Algae are identified as the microalgae, which are found in both benthic and littoral habitats and also throughout the ocean waters as phytoplankton and the macroalgae (seaweeds) which occupy the littoral zone. Phytoplankton comprises diatoms, dinoflagellates, green and yellow–brown flagellates, and blue–green algae while seaweeds are classified into green algae, brown algae, and red algae.

Marine algae are known to be a good source of healthy food due to their low content in lipids, high concentration in polysaccharides, natural richness in minerals, polyunsaturated fatty acids and vitamins. Especially, seaweeds are able to produce a great variety of secondary metabolites characterized by a broad spectrum of biological activities such anti-coagulation, anti-virus, anti-oxidant, anti-allergy, anti-cancer, anti-inflammation, and anti-obesity, anti-diabetes, anti-hypertension, neroprotection, immunomodulation [17–20]. Therefore, marine algae are believed to be a promising source to provide not only novel biologically active substances for the development of pharmaceuticals but also essential compounds for human nutrition [21].

The Phaeophyceae (brown algae) is a large group of marine multicellular algae, including of many seaweeds. They play an important role in marine environments, both as food and for the habitats they form. Although the division Phaeophyta consists of 13 orders according to the classification of Bold and Wynne [15], only three orders namely *Laminariales*, *Fucales*, and *Dictyotales* have been extensively researched for their phytochemicals. The most studied species of these orders are *Laminaria*, *Ecklonia*, *Undaria*, *Himanthalia*, *Sargassum*, and *Dictyota*. Brown seaweeds are rich in polysaccharide, polyphloroglucinol phenolic compounds, and other secondary metabolites such as terpenes, carotenoids, and oxylipins [21] Notably, marine brown algae accumulate a variety of phloroglucinol-based polyphenols, as phlorotannins. These pholorotannins consist of phloroglucinol units linked to each other in various ways, and are of wide occurrence among marine brown algae [22, 23]. Among marine

brown algae, *Ecklonia cava*, *Ecklonia stolonifera, Ecklonia kurome, Eisenia bicyclis, Ishige okamurae, Sargassum thunbergii, Hizikia fusiformis, Undaria pinnatifida,* and *Laminaria japonica* have been reported for phlorotannins with health beneficial biological activities [23]. This review focuses on phlorotannins from marine brown algae and presents an overview of their chemical properties as well as potential pharmaceutical applications.

11.2 Phlorotannins

11.2.1 Sources and Distribution

Phlorotannins have only been found to exist within brown algae and may constitute up to 15% of the dry weight of brown algae [24, 25]. The concentration of phlorotannins is highly variable among different brown seaweeds as well as among different geographical areas. The fucoid species from the Atlantic and the temperate Pacific contain higher concentration of phlorotannins as compared to those obtained from the tropical Pacific [26]. It was found that phlorotannnins have mostly focused on Fucaceae (*Ascophyllum nodosum* and *Fucus vesiculosus*), Sargassaceae (*Sargassum spinuligerum and Carpophyllum angustifolium*), and Cystoseiraceae (*Cystophora retroflexa and C. torulosa*) with concentrations ranging from 20 to 250 mg/g dry matter [27–31]. They tend to be concentrated within the outer cortical layers, physode, and the mitotic meristematic and meiotic sporogenous tissues [24, 32]. In addition, Laminariaceous brown algae, such as *Eisenia bicyclis, Ecklonia cava, Ecklonia kurome* were also found to contain a significant amount of phlorotannins [33, 34].

11.2.2 Structural Diversity and Classification

Phlorotannins are formed by the polymerization of phloroglucinol (1,3,5-trihydroxybenzene) monomer units. They are highly hydrophilic components with a wide range of molecular sizes ranging between 126 Da and 650 kDa. The monomeric units are linked through aryl-aryl bonds and diaryl ether bonds forming different subgroups of phlorotannins [35]. Phlorotannins can be grouped according to the criteria of interphloroglucinol linkages into three primary types including fucols, phlorethols, and fucophlorethols. Fucols is formed by only phenyl linkages, while phlorethols is formed by only arylether bonds and fucophlorethols is formed by both arylether and phenyl linkages.

The structural diversity of phlorotannins increases by adding the number of phloroglucinol units. Each of the primary groups can be grouped into linear phlorotannins, if all extension units have only two interphloroglucinol connections, or branched, if they bind to three or more. In fucols, the interphloroglucinol links at meta-relative position construct of the linear phlorotannin such as tetrafucol-A

and the branched phlorotannin such as tetrafucol-B, which were isolated from *Fucus vesiculosus* [36, 37].

Moreover, longer oligomers of phlorotannin such as pentafucols and heptafucols were purified from *Scytothamnus australis* [38] and *Analipus japonicas* [39]. The linear phlorethols may have ortho-, meta- or para- oriented biphenyl ether bridges or combinations such as triphlorethol C and tetraphlorethols A and B from *Laminaria ochroleuca* [40]. The branched phlorethols include tetraphlorethol C from *Ecklonia maxima* [41], pentaphlorethol B and hexaphlorethol A from *Cystophora retroflexa* [29].

Furthermore, an additional hydroxyl group on the terminal monomer unit forms other structural motifs of phlorethols such as bifuhalol, trifuhalol A, and trifuhalol B [42, 43]. If an extension unit is bound between meta-oriented phloroglucinol units and it bears the additional hydroxyl group, these are called isofuhalols such as isotrifuhalol [44]. Some fuhalols with more than one additional hydroxyl group have been called hydroxyfuhalols, such as hydroxytrifuhalol B [45]. In addition, another subgroup of phlorethols, the eckols, includes a 1,4-dibenzodioxin system, such as the trimers eckol and dioxinodehydroeckol [41, 46], the tetramers 2-phloroeckol and 7-phloroeckol [47–49], and the hexamer dieckol [50].

In fucophlorethols, the combinations of C–C and C–O–C linkages allow the formation of various compounds in linear, branched and heterocyclic fashions. The linear fucophlorethols is fucodiphlorethol-B [27], meanwhile the branched fucophlorethols is bisfucotriphlorethol A [51], and heterocyclic fucophlorethols is phlorofucofuroeckol A [52].

11.2.3 Biosynthesis of Phlorotannins

Phlorotannins are biosynthesized via the acetate-malonate pathway, also known as the polyketide pathway, in a process which may involve a polyketide synthase-type enzyme complex [53]. However, the exact biosynthetic pathway for phlorotannins is unknown up to now. Therefore, methodologies that monitor phlorotannin synthesis at the genetic or enzymatic levels could be useful to reveal some of the uncertainties regarding phlorotannin biosynthesis [54]. Firstly, two molecules of acetyl co-enzyme A are converted into malonyl co-enzyme A through the addition of carbon dioxide. This addition changes the acetyl methyl group into a highly reactive methylene. Secondly, the process of polymerization is assisted to occur with the low required energy. During further synthesis steps, the carbon dioxide, which was added as an activator, is lost. Thirdly, a polyketide chain consisting of an acid moiety is formed, and the co-enzyme is lost. The polyketide chain is transformed by intermolecular ring closure and elimination of water to produce hexacyclic ring systems. Triketide, the cyclization product, is not stable and thus undergoes transformation into the thermodynamically more stable aromatic form, phloroglucinol, consisting of three phenolic hydroxyl groups [55]. The polymerization of phloroglucinol in different ways results in formation of various phlorotannins.

11.2.4 Physiological Properties

Phlorotannins are found in physodes, which contribute to the development of the cell wall of brown algae [56]. It has suggested that phlorotannins are likely to be integral structural components of brown-algal cell walls [57]. They are bound to the cell wall during maturation of the plant [58]. Phenolic compounds are bound with four major types of bonds including hydrophobic, hydrogen, ionic, and covalent bond to increase the strength [59]. The cell wall (alginic acid) and phlorotannins are linked via covalent bonds including the ester bond and the hemiacetal bond, thus requiring strong conditions to degrade. Moreover, phlorotannins have a putative role in brown algal reproduction due to exposing on the surface of the recently fertilized zygotes where they may prevent multiple fertilizations by inhibiting spermatozoid movement [56].

A characteristic of phlorotannins is their plasticity to a variety of environmental factors including nutrient environment [60], light [61], depth [62], salinity [63], grazing [64] or other mechanical wounding [65]. Such plasticity may represent inducible defense against herbivory [25]. Suggestions for other adaptive roles for phlorotannins include protection against ultraviolet radiation [66] or function as anti-fouling substances [67]. The suggested defensive role of phlorotannins is due to deterring feeding by herbivores [68] and decreasing their assimilation efficiency by binding with proteins in the gut [69, 70].

11.3 Potential Health Benefits

11.3.1 Antioxidant and UV-Protective Activities

The oxidants such as superoxide anion radicals, hydroxyl radical species, and hydrogen peroxide are often generated by biological oxidation reactions of exogenous factors [71]. It is well known that oxidants are involved in signal transduction and gene activation, and can contribute to host cell and organ damage [72]. Therefore, scavenging of oxidant is considered important in controlling various diseases. Interestingly, phlorotannins from marine brown algae have been evidenced to be effective to scavenge oxidants in non-cellular and cellular systems. According to Ahn and colleagues, the antioxidant activities of three phlorotannins including phloroglucinol, eckol and dieckol purified from *Ecklonia cava* collected in Jeju Island have been investigated [73]. It reported that all the phlorotannins have the potential DPPH, alkyl, hydroxyl and superoxide radical scavenging activities. Eckol exhibit the most strong antioxidant activity via scavenging 93% of DPPH. Moreover, these phlorotannins were effective to protect DNA against H_2O_2-induced damage.

In the same trend, Kang and colleagues have also investigated the cytoprotective effect of eckol from *E. cava* against oxidative stress induced cell damage in Chinese hamster lung fibroblast (V79-4) cells [74]. Eckol was effective to reduce H_2O_2-

induced cell death in V79-4 cells, inhibit radiation-induced cell damage, and scavenge intracellular ROS production. Moreover, eckol was able to increase the activity of catalase and its protein expression via increasing phosphorylation of extracellular signal-regulated kinase and activity of nuclear factor κB. In another study by Kang et al. triphlorethol-A from *E. cava* was found to reduce intracellular hydrogen peroxide generated by gamma-ray radiation, thus protecting against radiation-induced membrane lipid peroxidation, cellular DNA damage, and cell death [75]. Furthermore, triphlorethol-A augments cellular antioxidant defense capacity through induction of HO-1 expression via ERK-Nrf2-ARE signaling pathway, thereby protecting cells from oxidative stress [76].

Notably, Li and colleagues have isolated several phlorotannins from *E. cava* such as phloroglucinol, eckol, fucodiphloroethol G, phlorofucofuroeckol A, dieckol, and 6, 6′-bieckol. All phlorotannins were found to possess antioxidant properties via scavenging free radicals, protecting membrane protein from oxidant-induced damage, enhancing cellular glutathione level in RAW264.7 cell line [77]. Likewise, several phlorotannins including phloroglucinol, eckol, dieckol, eckstolonol and triphloroethol A from *E. cava* were investigated for their activity against AAPH-induced oxidative stress toxicity in zebrafish embryos [78]. All phlorotannins were able to scavenge intracellular ROS, prevent lipid peroxidation and reduce AAPH-induced cell death in zebrafish embryos. In an in vivo study, the role of eckol from *E. cava* as a radioprotective agent against the gamma ray-induced damage has been investigated by Park et al. [79]. It has been determined that eckol significantly decreased the mortality of lethally irradiated mice via improving the hematopoietic recovery, repairing the damaged DNA in immune cells and enhancing their proliferation. Therefore, eckol is considered as a potential candidate for adjuvant therapy of radiation-exposed cancer patients.

UV radiation has a strong oxidative component, and photo-oxidative stress has been directly linked to skin photodamage, and associated with abnormal cutaneous reactions such as epidermal hyperplasia, accelerated breakdown of collagen, and inflammatory responses. Herein, dieckol from *E. cava* has been found to be able to inhibit melanogenesis and protect against photo-oxidative stress induced by UV-B radiation [80]. The inhibitory activity on melanogenesis was evidenced via suppression of tyrosinase and melanin synthesis. Meanwhile, protective activity was observed via scavenging intracellular ROS, preventing DNA damage, and increasing cell viability. Additionally, Fucofuroeckol-A from *E. stolonifera* was also found as protective agent against UVB-induced allergic reaction in RBL-2H3 mast cells [81]. It was revealed that F-A significantly suppress mast cell degranulation via decreasing histamine release as well as intracellular Ca^{2+} elevation induce by UVB. Notably, the protective activity of F-A against mast cell degranulation was found due to scavenging ROS production. These results indicated that phlorotannins from brown algae have potential protective effects against UV-B radiation, which might be applied in cosmeceutical industries.

11.3.2 Antimicrobial Activity

Infectious diseases caused by bacteria and fungi are still a major threat to public health, despite the tremendous progress in human medicine. Increasing resistance of clinically important bacteria to existing antibiotics is a major problem throughout the world [82]. The discovery of novel antimicrobial compounds for clinical application is necessary to check the global crisis of antibiotic resistance. In this regard, phlorotannins from brown algae have been found to possess antimicrobial effect against food-borne pathogenic bacteria, antibiotic resistance bacteria, and pathogenic fungi. According to Nagayama and colleagues, the oral administration of phlorotannins from *E. kurome* on mice results in effective inhibition against methicillin-resistant *Staphylococcus aureus* (MRSA). The minimum bactericidal concentrations (MBCs) of eckol, phlorofucofuroeckol A, dieckol, and 8,8′-bieckol against *Campylobacter jejuni* were 0.08, 0.08, 0.03, and 0.03 μmol/ml, respectively. At twice the MBCs, all *Vibrio parahaemolyticus* were killed within 0.5–2 h, while catechins showed little bactericidal activity within 4 h [83]. Furthermore, Lee and co-workers have determined that dieckol from *E. stolonifera* exhibited antibacterial activity against methicillin-susceptible S. aureus (MSSA) and MRSA in a range of minimum inhibitory concentrations (MICs) of 32 to 64 μg/ml [84]. The MICs of ampicillin against two standard strains of MRSA were dramatically reduced from 512 to 0.5 μg/ml in combination with 1/4 MIC of dieckol (16 μg/ml). Likewise, Phlorofucofuroeckol-A from *E. bicyclis* were also showed anti-MRSA activity with MIC of 32 μg/ml and synergistic action against MRSA in combination with β-lactam antibiotics ampicillin, penicillin, and oxacillin [85]. Thereby, phlorotannins-β-lactam antibiotics combinations exert a synergistic effect against MRSA, indicating the promising treatment of MRSA infections. In addition, it has shown that phlorofucofuroeckol-A from *E. cava* and *E. bicyclis* exhibited effective inhibition against *Propionibacterium acnes*, which may be useful as natural additives in anti-acne cosmetic products [86, 87]. Although the relationship between the structure and anti-bacterial activity of the phlorotannins is limited, their inhibitory activity may be suggested to depend on the degree of polymerization of phlorotannin derivatives.

Besides, the purified phlorotannins extracts from three brown seaweeds including *Cystoseira nodicaulis*, *C. usneoides*, and *Fucus spiralis* displayed their antifungal activity against human pathogenic yeast and filamentous fungi [88]. It was revealed that *C. albicans* ATCC 10231 was the most susceptible among yeast, while *Epidermophyton floccosum* and *Trichophyton rubrum* were the most susceptible among dermatophytes. It was found that *C. nodicaulis* and *C. usneoides* seem to act by affecting the ergosterol composition of the cell membrane of yeast and dermatophyte, respectively. Meanwhile, *F. spiralis* influenced the dermatophyte cell wall composition by reducing the levels of chitin. Moreover, phlorotannins from *F. spiralis* inhibited the dimorphic transition of *Candida albicans*, leading to the formation of pseudohyphae with diminished capacity to adhere to epithelial cells. On the other hand, the potential fungicidal activity of dieckol from *E. cava* was also found due to

inhibition of *Trichophyton rubrum* associated with dermatophytic nail infections in human [89].

11.3.3 Anti-HIV Activity

Human immunodeficiency virus type-1 (HIV-1) is the cause of acquired immune deficiency syndrome (AIDS) which has been a major human viral disease with about 33.2 million people infected worldwide up to now [90, 91]. Antiviral agents that interfere with HIV at different stages of viral replication have been developed [92, 93]. However, failure in anti-AIDS treatment is observed by the emergence of resistant virus, cross-resistance to drugs and cell toxicity [94, 95]. Therefore, the search for potential candidates containing higher inhibitory activity against various HIV strains is increasing in pharmaceutical industry. Accordingly, phlorotannins from brown algae have been revealed to possess anti-HIV activity.

For the first time, Ahn et al. [96] reported that 8,8′-bieckol and 8,4′′-dieckol from *E. cava* exhibited an inhibitory effect on HIV-1 reverse transcriptase and protease. The inhibition against reverse transcriptase of 8,8′-bieckol with a biaryl linkage (IC_{50}, 0.5 μM) is ten-fold higher than that of 8,4′′-dieckol with a diphenyl ether linkage (IC_{50}, 5.3 μM), although these two phlorotannis are dimmers of eckol. They have suggested that the steric hindrance of the hydroxyl and aryl groups near the biaryl linkage of 8,8′-bieckol caused to the potent inhibitory activity. Moreover, 8,8′-bieckol selectively inhibits reverse transcriptase over protease and inhibitory effect is comparable to the positive control nevirapine (IC_{50}, 0.28 μM). Moreover, kinetic study showed that 8,8′-bieckol inhibited the RNA-dependent DNA synthesis activity of HIV-1 reverse transcriptase noncompetitively against dUTP/dTTP with a Ki value of 0.78 μM. Meanwhile, this compound also exhibited an uncompetitive inhibition (Ki, 0.23 μM) with respect to a homopolymeric template/primer, $(rA)_n(dT)_{15}$. A possible suggestion for this phenomenon is that 8,8′-bieckol binds to HIV-1 reverse transcriptase only after the template/primer initially binds to the enzyme. Furthermore, their study has also revealed shown that diphlorethohydroxycarmalol from *I. okamurae* also has inhibitory effect on HIV-1 [97]. This compound exhibited inhibitory effects on HIV-1 reverse transcriptase and integrase with IC_{50} values of 9.1 μM and 25.2 μM, respectively. However, diphlorethohydroxycarmalol did not show an inhibitory activity against HIV-1 protease.

In the same trend, 6,6′-bieckol from *E. cava* has been found as a potent wild inhibition against HIV-1 induced syncytia formation, lytic effects, and viral p24 antigen production [98]. This phlorotanins has selectively inhibited the activity of HIV-1 reverse transcriptase enzyme with an IC_{50} of 1.07 μM without any cytotoxicity. Recently, Kwon and colleagues have found that phlorotanins including eckol, 7-phloroeckol, phlorofucofuroeckol, and dieckol possessed antiviral activities with IC_{50} range of 10.8–22.5 μM against porcine epidemic diarrhea virus [99]. These phlorotanins were completely blocked binding of viral spike protein to sialic acids at less than 36.6 μM by hemagglutination inhibition. Notably, phlorofucofuroeckol

and dieckol inhibited viral replication with IC_{50} values of 12.2 and 14.6 μM in the post-treatment assay, respectively. Interestingly, phlorofucofuroeckol and dieckol inhibited both viral entry by hemagglutination inhibition and viral replication by inhibition of viral RNA and viral protein synthesis, but not viral protease.

11.3.4 Anti-allergic Activity

Allergic disease including allergic rhinitis, asthma, and atopic eczema are among the commonest causes of chronic ill health. It is caused by an exaggerated reaction of the immune system to harmless environmental substances, such as animal dander, house dust mites, foods, pollen, insects, and chemical agents [100, 101]. Allergic reaction is characterized by the excessive activation of mast cells and basophils by immunoglobulin E (IgE) from B cells, resulting in the release of preformed inflammatory mediators from secretory granules such as histamine and β-hexosaminidase, the generation and secretion of the newly synthesized substances such as leukotrienes, prostaglandins, and cytokines [102]. These mediators cause allergic inflammatory responses due to airway constriction, mucous production, and recruitment of inflammatory cells. So far, a large number of anti-allergic agents from natural products have been identified based on the specific assay system or screening approaches.

Recently, phlorotannins from brown algae have been determined as potential natural inhibitors of allergic reactions due to suppression of allergic degranulation, inhibition of hyaluronidase enzyme, and blockade of FcεRI activities. Several bioactive phloroglucinol derivatives including fucodiphloroethol G, eckol, dieckol, 6, 6′-bieckol, phlorofucofuroeckol A, and 1-(3′,5′-dihydroxyphenoxy)-7-(2″,4″,6-trihydroxyphenoxy)-2,4,9-trihydroxydibenzo-1,4-dioxin were isolated from *E. cava* and evidenced against A23187 or FcεRI-mediated histamine release from KU812 and RBL-2H3 cells [34, 103]. Especially, dieckol, 6,6′-bieckol, and fucodiphloroethol G exhibited a significantly inhibitory activity with IC_{50} range of 27.80–55.12 μM. The inhibitory mechanism of these compounds was determined to be due to blocking the binding activity between IgE and FcεRI. Similarly, Shim et al. [104] have proved that phlorotannins of dioxinodehydroeckol and phlorofucofuroeckol A from *E. stolonifera* induced a suppression of the cell surface FcεRI expression, and total cellular protein and mRNA levels of the FcεRI α chain in KU812 cells. Further, both of these compounds exerted inhibitory effects against intracellular calcium elevation and histamine release from anti-FcεRI α chain antibody (CRA-1)-stimulated cells. In another study, phlorotannin PFF-B obtained from *E. arborea* exposed strong inhibitory activity against histamine and β-hexosaminidase release with IC_{50} value of 7.8 μM [105, 106]. Obviously, PFF-B had a 2.8–6.0 times greater inhibitory activity than those of epigallocatechin gallate (IC_{50} = 22.0 μM) or Tranilast (IC_{50} = 46.6 μM), a clinically used anti-allergic drug [107]. Thus, these bioactive phloroglucinol derivatives were suggested as a promising candidate for the design of novel inhibitor of FcεRI-mediated allergic reaction.

Hyaluronidase depolymerizes the polysaccharide hyaluronic acid in the extracellular matrix of connective tissue, which is found both in organs and in body fluids. It is mainly known to be involved in the permeability of the vascular system [108] and allergic reaction [109, 110]. Interestingly, various phlorotanins such as phlorofucofuroeckol A, dieckol, and 8,8′-bieckol from *E. bicyclis* are able to inhibit hyaluronidase enzyme with IC_{50} values of 140, 120, and 40 μM, respectively [111]. The effect of these phlorotannins against hyaluronidase enzyme is stronger than well-known inhibitors such as catechins ($IC_{50} = 620$ μM) and sodium cromoglycate ($IC_{50} = 270$ μM). Notably, 8,8′-bieckol, the strongest hyaluronidase inhibitor among the tested phlorotannins, acted as a competitive inhibitor with an inhibition constant of 35 μM. Likewise, several phlorotannins of 6,6′-bieckol, 6,8′-bieckol, 8,8′-bieckol, PFF-A, and PFF-B from *E. arborea* were also confirmed as strong inhibitors of hyaluronidase [112, 113].

11.3.5 Anti-inflammatory Activity

Inflammation is a critically important aspect of host responses to various stimuli including physical damage, ultra violet irradiation, microbial invasion, and immune reactions [114, 115]. It is associated with a large range of mediators that initiate the inflammatory response, recruit and activate other cells to the site of inflammation [116]. However, excessive or prolonged inflammation can prove harmful, contributing to the pathogenesis of a variety of diseases, including chronic asthma, rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease, psoriasis, and cancer [115]. Currently, several classes of drugs such as corticosteroids, nonsteroidal anti-inflammatory drugs, and aspirin are used to treat the inflammatory disorders. All these therapeutics help to alleviate the symptoms but, especially after long-term and high-dose medication, they can have quite substantial side effects. Therefore, there is still a vital need for the development of new anti-inflammatory drugs with satisfactory tolerability for long-term use. Herein, phlorotannins have been evidenced as potential agents for down-regulation of inflammatory responses. Phlorotannin-rich extracts of *E. cava* showed significant suppression of PGE_2 generation in LPS-treated RAW 246.7 cells, and significant inhibition of human recombinant interleukin-1α-induced proteoglycan degradation [117]. Moreover, the phlorotannin-rich the fermented *E. cava* processing by-product extract was reported to inhibit NO and PGE_2 production, suppress the inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) expressions, and attenuate interleukin-1β and interleukin-6 production in lipopolysaccharide stimulated RAW 264.7 cells [118]. Additionally, pretreatment of phlorotannin-rich extracts of *Ascophyllum nodosum* caused reduction of LPS-induced TNF-α and IL-6 release in macrophages [119]. Recently, phlorotannin 6,6′-bieckol from *E. cava* was found to inhibit NO and PGE_2 production by suppressing the expression of iNOS and COX-2 at the mRNA and protein levels in LPS-stimulated primary macrophages and RAW 264.7 macrophage cells [120]. Moreover, 6,6′-bieckol down-regulated the production and mRNA expression of the inflammatory

cytokines TNF-α and IL-6. The pretreatment of 6,6′-bieckol decreased LPS-induced transactivation of nuclear factor-kappa B (NF-κB) and nuclear translocation of p50 and p65 subunits of NF-κB, thus inhibiting LPS-induced NF-κB binding to the TNF-α and IL-6 promoters. On the other hand, Kim and collaborators have evidenced that phlorofucofuroeckol A from *E. stolonifera* attenuated the productions and expression of NO, PGE_2, and pro-inflammatory cytokines such as IL-1β, IL-6, and TNF-α in LPS-stimulated microglia. Profoundly, phlorofucofuroeckol A treatment showed inactivation of c-Jun NH2-terminal kinases (JNKs), p38 mitogen-activated protein kinase (MAPK), Akt, and NF-κB [121]. Similar observations were also made in their earlier study related to the inhibitory activity of this phlorofucofuroeckol A on NO and PGE_2 production and iNOS and COX-2 expression in RAW 264.7 murine macrophage cells [122]. Besides, phlorotanins from *E. arborea* also exhibited inhibitory effect on NO production in LPS-stimulated RAW 264.7 cells [123] and mouse ear edema induced by arachidonic acid, 12-O-tetradecanoyl phorbol-13-acetate, and oxazolone [124]. Notably, 8,8′-bieckol from *E. bicyclis* showed the pronouncedly inhibitory effects on soybean lipoxygenases and 5-lipoxygenases with IC_{50} values of 38 and 24 μM, respectively. Meanwhile, dieckol presented a significant inhibition of COX-1 with inhibition rate of 74.7% [125]. Similarly, 6,6′-bieckol, 6,8′-bieckol, 8,8′-bieckol, PFF-A, and PFF-B from *E. arborea* were also confirmed as strong inhibitors of phospholipase A_2, cyclooxygenase, and lipoxygenases, which correlated to suppression in synthesis and release of leukotoriene and prostaglandin from RBL cells [113].

11.3.6 Anti-cancer Activity

Cancer can be defined as a disease in which a group of abnormal cells grows uncontrollably by disregarding the normal rules of the cell division [126]. Cancers may be caused in one of three ways, namely incorrect diet, genetic predisposition, and via the environment. At least 35% of all cancers worldwide are caused by an incorrect diet. Meanwhile, genetic predisposition caused about 20% of cancer cases, thus leaving the majority of cancers being associated with a host of environmental carcinogens [127]. It is necessary to avoid exposure to cancer-causing biological, chemical, and physical agents, and consume chemopreventive agents to reduce cancer risk. A promising approach is associated with natural products that are available as anti-cancer agents against commonly occurring cancers occurring worldwide [128, 129]. Recently, phlorotannins have been reported as novel promising anti-cancer agent for breast cancer. Kong et al. [130] has indicated that dioxinodehydroeckol from *E. Cava* exerted anti-proliferative activity against human breast cancer cells via induction of apoptosis. Dioxinodehydroeckol treatment caused the increase in caspase (−3 and −9) activity, DNA repair enzyme poly-(ADP-ribose) polymerase (PARP) cleaved, and pro-apoptotic gene (Bax, p53, and p21) and the decrease in anti-apoptotic gene Bcl-2 and NF-κB activation. Moreover, phlorotannins-rich extracts from *Palmaria*, *Ascophyllum* and *Alaria* also inhibited the proliferation of colon cancer cells [131].

On the other hand, the anti-cancer activity of *S. muticum* polyphenol-rich seaweed was shown via inhibiting the proliferation of breast cancer cells with IC_{50} of 22 μg/ml and inducing apoptosis from 13 to 67% by accumulation of cells at sub-G1 phase [132]. Parys et al. [133] reported that trifucodiphlorethol A, trifucotriphlorethol A and fucotriphlorethol A from *Fucus vesiculosus* were the potential chemopreventive agents due to their capacity to inhibit the activity of aromatase related to carcinogenesis from breast cancers. For the first time, Kim and colleages have determined the inhibitory effects of phlorotannins isolated from E. cava on MMP activities in cultured human cell lines without any cytotoxic effect [134].

11.3.7 Anti-diabetic Activity

Diabetes mellitus is a chronic metabolic disorder involved in hyperglycaemia, resulting from the deficiency in the production of insulin by the pancreas. Up to now, numerous therapeutics has been proposed to control hyperglycaemia in diabetic patients. Especially, α-amylase and α-glucosidase are enzymes related to hyperglycaemia due to the starch hydrolysis and release of the glucose monomers for subsequent absorption by the small intestine. Therefore, the inhibition of these enzymes reduces the availability of free glucose monomers and consequently decreases blood glucose levels [135]. Rengasamy et al. [136] has isolated three phlorotannins including dibenzo (1,4) dioxine-2,4,7,9-tetraol and eckol from *E. maxima* and evaluated their alpha-glucosidase inhibitory activities. The inhibitory activities of dibenzo (1,4) dioxine-2,4,7,9-tetraol and eckol on enzyme alpha-glucosidase were 33.7 and 11.2 μM, respectively. A phenolic-rich extract from *Ascophyllum* was effective to inhibit α-amylase and α-glucosidase with IC_{50} of 0.1 μg/ml GAE and 20 μg/ml GAE [131]. The presence of fucophloroethol structures with degrees of polymerization from 3 to 18 monomer units in *Fucus distichus* is responsible for its inhibition on α-glucosidase and α-amylase, with IC_{50} values of 0.89 and 13.9 μg/ml [137]. Moreover, dieckol and eckol from *Eisenia bicyclis* exhibited the inhibitory activity on α-amylase up to 97.5 and 87.5% at 1 mM [138]. Meanwhile, α-glucosidase was inhibited by phlorofucofuroeckol-A, dieckol, and 7-phloroeckol from *E. stolonifera* and eckol and dioxinodehydroeckol from *E. bicyclis* with IC_{50} of 1.37, 1.61, 6.13, 22.78, and 34.6 μM, respectively [139]. The ingestion of methanolic extract of *E. stolonifera* suppressed the increase in plasma glucose and lipid peroxidation levels in unfasted KK-A(y) mice [140]. Furthermore, various phlorotannins from *E. stolonifera* exhibited the inhibitory activities on aldose reductase, which are highly implicated in hyperglycemia and oxidative stress. The IC_{50} values of phloroglucinol derivatives are 21.95–125.45 μM [141]. Besides, dieckol from *E. cava* has evidenced prominent inhibitory effect against alpha-glucosidase and alpha-amylase with IC_{50} values of 0.24 and 0.66 mM, respectively. The increase of postprandial blood glucose levels was significantly suppressed in the dieckol administered group in the streptozotocin-induced diabetic mice [142]. Recently, three phlorotannins, eckol, dieckol and phlorofucofuroeckol-A from *E. bicyclis* were revealed for their anti-

diabetic activity of alloxan-induced type1 and insulin-induced type 2 in the zebrafish model [143].

11.3.8 Anti-obesity

Obesity is a major obstacle in human health and life quality, resulting in many chronic diseases. It is due to a chronic imbalance between energy intake and energy expenditure, leading to the increased fat storage [144]. Interestingly, a series of anti-obesity components derived from marine origin have been found, especially phlorotannins. Herein, three phlorotannins from *E. stolonifera* including phloroglucinol, eckol, and phlorofucofuroeckol A significantly inhibited lipid accumulation in 3T3-L1 cells via reducing the expression of adipocyte marker genes such as proliferator activated receptor γ and CCAAT/enhancer-binding protein α [145]. Meanwhile, phlorotannin dieckol from *E. cava* exhibited great potential adipogenesis inhibition and downregulated the expression of peroxisome proliferator-activated receptor-γ, CCAAT/enhancer-binding proteins, sterol regulatory element-binding protein 1 (SREBP1) and fatty acid binding protein 4 [146]. Moreover, diphlorethohydroxycarmalol (DPHC) from *Ishige okamurae* was showed to inhibit population growth and induce apoptosis in 3T3-L1 preadipocytes [147]. The peptidyl prolyl cis/trans isomerase Pin1 enhances the uptake of triglycerides and the differentiation of fibroblasts into adipose cells in response to insulin stimulation. However, phlorotannin called 974-B from *E. kurome* was showed to inhibit the differentiation of mouse embryonic fibroblasts and 3T3-L1 cells into adipose cells without inducing cytotoxicity, suggesting a lead drug candidate for obesity-related disorders [148].

11.3.9 Other Biological Activities

According to Ahn et al. [149], phloroglucinol from *E. cava* possesses the activation activity on immune response. The phloroglucinol elicited the proliferation of lymphocytes without cytotoxicity and enhanced IL-2 production by activating the nuclear factor-kappaB (NF-κB) signaling pathway.

Inhibition of angiotensin I-converting enzyme (ACE) activity is the most common mechanism underlying the lowering of blood pressure. Dieckol from *E. cava* was found as potent ACE inhibitor with IC_{50} value of 1.47 mM. It is a non-competitive inhibitor against ACE according to Lineweaver-Burk plots [150]. Meanwhile, eckol, phlorofucofuroeckol A, and dieckol from *E. stolonifera* were also determined to manifest the marked inhibitory activity against ACE, with IC_{50} values of 70.82, 12.74, and 34.25 μM, respectively [151].

11.4 Conclusion

Finding the safe and efficient agents from natural products for prevention and treatment of chronic diseases are always necessary. Herein, phlorotannins from brown algae have been identified with various biological activities and health benefit effects. The extensive discoveries of phlorotannins underlying structure-activity relationship will provide a clear evidence on their actions against diseases. Moreover, the further studies due to the bioavailability involving in liberation, absorption, distribution, metabolism, and elimination phases will ensure the bioefficacy of phlorotannins. Collectively, phlorotannins from brown algae are believed to play an important role in the development of novel products that can prevent and/or treatment of chronic diseases.

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Part III
Marine Sources for Tissue Engineering Scaffolds

Chapter 12
Sponge (Porifera) Collagen for Bone Tissue Engineering

Ming-Hao Zheng and Jessica Zheng

Abstract The ultimate goal of tissue engineering is to regenerate and/or replace fully functional tissue, or to stimulate the body to regenerate its own fully functional tissue (Vacanti and Langer in Lancet 354:SI32–SI34, 1999). This technology is of particular use in orthopaedics where various reconstructive operations are conducted throughout the musculoskeletal system. Many tissue engineering techniques utilize specific combinations of living cells, manufactured macromolecular biomaterials (matrices), and bioactive factors (cytokines and/or growth factors) to direct synthesis and organization of tissues (Fodor in Reproductive Biology and Endocrinology 1:102, 2003). The architecture and biochemical nature of matrices is a key aspect of cell-based tissue engineering. The matrix provides a vehicle for delivery of stem cells and progenitors to a desired site, and provides surfaces that facilitate the attachment, survival, migration, proliferation and differentiation of these cells. The ideal scaffold requirements for bone tissue engineering include biocompatibility, osteoconductive or osteoinductive capacity, high porosity that enables nutrient transport, infiltration of cells, degradability over suitable time scales, and interstitial flow of fluid (Bruder and Fox in Clinical Orthopaedics and Related Research 367:S68–83, 1999). The skeletons of Porifera appear to have unique properties that may provide for potential bioscaffolds in cell-based bone tissue engineering. These properties include the collagenous composition of the fiber skeleton, its ability to hydrate to a high degree, and the possession of open interconnected channels created by the fiber network (Green et al. in Tissue Engineering 9:1159–1166, 2003). In addition to this, the phylum has a tremendous diversity of skeletal architecture within the 8000 extant species currently described, many of which are readily available for use (Hooper and Van Soest in Systema Porifera: a guide to the classification of sponges. Academic/Plenum, New York, 2002).

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12.1 Introduction

The skeletal system forms the scaffold of the human body—it provides the basis of support for our structure and is vital for human function. Bone consists of not only bone tissue, but also various types of connective tissues, including adipose tissue, hemopoietic tissue, blood vessels and nerves [6]. Bone tissue is a highly dynamic, specialized connective tissue comprising a mineralized extracellular matrix (ECM) embedded with bone cells, blood vessels, and nerves. The ECM consists of predominately hydroxyapatites, type I collagen, and ground substance that contains proteoglycans, and non-collagenous glycoproteins. The collagen is secreted by osteoblasts initially as stacks of polypeptide molecules arranged in a helical fashion. Crystals of minerals are deposited on the collagen molecules as they are being formed into fibrils and fibres, which produce extremely hard tissue that provides important support and protection. This mineral is calcium phosphate, which deposits in the form of hydroxyapatite crystals [$Ca_{10}(PO_4)_6(OH)_3$] [7].

Over two million bone-grafting procedures are performed worldwide each year—the second most frequent type of tissue transplantation. Traditional procedures in bone tissue grafting may include the use of grafts such as autografts (harvesting the patient's own bone from one site and transferring it to another site), allografts (cadaver bone, washed to remove most cells and often frozen), alloplasts (synthetic materials that serves as a bone-forming scaffold), or xenografts (naturally derived materials that serve as a bioscaffold). Surgical invasion in the setting of autografts, implants and prosthetics, comes with a range of acute and chronic issues, including pain, infection, thrombotic and bleeding complications, failure to cure and repeat surgery. Autografts may also lead to donor-site morbidity. Alternatively, allografts may be used, however these lack the osteoactive capacity of autografts, and have the additional risk of infection and immune rejection [8]. Tissue engineering has the potential to treat damaged or diseased tissues, and eliminate these complications, and therefore holds tremendous potential in creating alternatives to harvested tissues, implants and prostheses [9]. The ultimate goal of bone tissue engineering is to regenerate and/or replace functional tissue, or to stimulate the body to regenerate its own fully functional tissue [1, 10].

12.2 Design Criteria of an Ideal Scaffold for Bone Tissue Engineering

A key element in bone tissue engineering is the use of a scaffold. Ideal scaffold requirements for bone tissue regeneration include biocompatibility, osteoconductive or osteoinductive capacity, high porosity (with large interconnected pores) that enables nutrient transport, infiltration of cells, degradability over suitable time scales, and interstitial flow of fluid [3]. These scaffolds are used as a vehicle for delivery of stem cells and progenitors to a desired site, as they are able to facilitate cell attachment, migration, viability, proliferation, and differentiation of these cells. They also provide a structural basis in which neovascularization, load bearing, host integration and formation of new tissue can occur [11]. One of the challenges in using scaffolds for bone tissue engineering is the matching of mechanical properties to the implant environment. Another challenge is to provide the scaffold with surface chemistry and topography that is conducive to the cell's natural environment [12]. Surface chemistry and topography can either be created by functionalizing the surface of the matrix with elements of the tissue's natural extracellular matrix, or alternatively, by utilizing naturally derived extracellular matrices that retain their own growth factors [13].

Matrix architecture defines the mechanical structure of the scaffold, as well as the initial space that allows for connective tissue progenitors to form new tissue, such as blood vessels and pathways for mass transport [13]. Matrix designs are unlimited, and involve numerous different macrostructures such as geometric shapes, amorphous structures and randomly integrated structures [14]. The physical structure and design of a scaffold depends on its application and the site of its designated implantation. Scaffolds may be three-dimensional, such as plugs, rods, or screws; this three-dimensional nanostructure has important effects on cell behavior as it allows for the diffusion of soluble materials [13, 15, 16]. It has been observed that osteoblasts respond and adhere well to nanophase materials (materials with grain sizes less than 100 nm), however, the mechanisms in which this occurs is yet to be fully understood. It is hypothesized that nanostructural features control protein interactions such as adsorption, configuration, and bioactivity [17, 18]. Nanoscale surface topographies create an ideal scaffold environment as they allow for favorable matrix binding sites for cell regulation and behavior, whilst also interacting with the host cells [12].

Pore dimension and interconnectivity are key factors in the structural design of tissue engineering scaffolds. There is a fine balance to optimizing the porosity of a scaffold; ideally, it should be large enough to allow for maximal invasion of cells necessary for reconstruction of the tissue, nutrient diffusion and removal of waste products, whilst also taking into consideration a high porosity will decrease surface

area for cell attachment and proliferation [19, 20]. This feature should also provide conduits suitable for blood vessel anastomosis [21].

Green et al. [22] found that osteoblasts respond directly to the pore dimensions of scaffold frameworks. It has been shown that pore diameters between 15 and 50 μm induce fibrovascular growth, whereas those between 50 and 150 μm stimulate osteoid formation [13]. Pore dimensions of 150–500 μm have been found to lead directly to mineralized bone, and also limit cell aggregation along the scaffold edge, therefore are used for most bone engineering scaffolds [22, 23]. In all settings in which cells are transplanted, access to substrate molecules (oxygen, glucose, and amino acids) and clearance of products of metabolism (CO_2, lactate, and urea) are critical to cell survival [19, 20]. In most tissues, passive diffusion along concentration gradients is the principal mechanism for mass transport, particularly for small molecules [24, 25].

In the case of transplantation scaffolds, diffusion distance is critical. Oxygen and nutrients become limited towards the center of the transplant; therefore, the size and interconnected porosity of the scaffold are important factors to consider. In situations (e.g. clinical grafts) where diffusion distance from the outer edge to the center is greater than in the natural environment, diffusion is only able to support a limited number of transplanted cells before metabolites accumulate in the center of the graft, resulting in central necrosis [26]. Survival of seeded cells also depends on the rate and extent of revascularization, which is in turn dependent on the size, porosity, and complexity of the scaffold. The vascularization of a scaffold occurs via capillary ingrowth from the host tissue into the open pore network of the scaffold [27]. The distance between blood vessels and mesenchymal cells in situ is no larger than 100 μm [28], therefore, the time taken for capillary systems to distribute through the scaffold must be considered when designing the size, porosity, and shape of the scaffold, as well as concentration of cells transplanted (as cell-seeding density influences initial spatial distribution of cells) [27, 29]. This is of vital importance as studies have shown that fast revascularization favors osteoblastic differentiation, whereas prolonged hypoxia favors formation of cartilage or fibrous tissue [24, 26].

Mechanical and physical stress exerted on the scaffold or tissue implant influences regenerative healing processes as well as tissue adaptation to new environments [30]. Mechanical properties such as strength, durability and ductility are determined by the scaffold's material properties and structure; the individual load bearing capacity of a scaffold is largely dependent on material type. For example, scaffolds needing to bear heavy loads following implantation may require high strength materials such as ceramic or metal [13].

A hypothesis in the design of tissue engineering scaffolds is that the scaffold should provide a biomimetic mechanical environment for remodelling and regenerating tissue, whilst simultaneously providing adequate porosity for cell migration [31]. However, in many cases this presents contradictory scaffold design require-

ments. In the case of bone tissue engineering, to match tissue stiffness and strength, the scaffold would need to be a dense material, however, when matching cell migration, a more porous scaffold composition is required [31, 32]. Yaszemski et al. [33] stated that bone tissue engineering scaffolds should possess mechanical stiffness matching the low-range of trabecular bone stiffness, 50–100 MPa. However, Hutmacher proposed that scaffold should match the native tissue stiffness, which for trabecular bone can range from 10 to 1500 MPa [27, 34].

Scaffolds used in regeneration of bone tissues need to have a high elastic modulus and provide the tissue with adequate space for tissue development in order to be retained in their designated implantation site [27]. Temporary load-bearing devices should also provide sufficient support to withstand in vivo forces until the engineered tissue has sufficient mechanical integrity to support itself [35]. In an effort not to place limitations on the success of tissue healing before regeneration, matching mechanical properties of a scaffold to the implant environment is vital. In most cases, the degradation of the scaffold must parallel the regeneration of tissue.

During tissue regeneration, as the cells begin to secrete their own extracellular matrix, the scaffold should degrade, and eventually eliminate from the body, resulting in complete natural tissue replacement [24]. Therefore, a challenge in matrix design is the control of the mechanical properties of the scaffold, and their degradation over time. The scaffold must have the required load-bearing strength, however at the same time must have a chemical and physical structure that allows for hydrolytic attack and breakdown [27]. Subsequently, the majority of scaffolds being developed ultimately resorb as defined by the regenerative requirements of the tissue targeted for treatment [13, 36, 37].

12.3 Natural Scaffolds for Tissue Engineering

Although a wide variety of scaffolding materials are available, it is important in tissue engineering to select a material that closely matches the properties of the tissue it seeks to replace [38]. Natural scaffolds offer better biocompatibility and bioactivity, whilst synthetic polymers enable the manipulation of physiochemical properties such as porosity, microstructure, degradation rate, and mechanical properties [39]. Collagen, glycosaminoglycan-based materials, chitosan, and alginates are examples of natural materials used in tissue engineering, of which collagen is the most widely used natural polymer in bone and cartilage tissue engineering [38, 39].

As natural bioscaffolds provide a microenvironment onto which cells can attach and migrate, theoretically, they should not cause a significant foreign body reaction when implanted. They therefore preclude many of the complications associated

with synthetic scaffolds such as biodegradability, poor biocompatibility and tissue rejection [40, 41]. In addition to inherent cell compatibility, natural bioscaffolds can also be processed in such a way that they retain their structure and composition, as well as growth factors [22]. Biological tissues possess the strength of the tissues from which the materials are derived, however, they are comparatively weaker than many synthetic scaffolds and can be degraded by naturally occurring enzymes [38]. Natural materials may be combined into a composite with other natural or synthetic materials, thus yielding the mechanical strength of the synthetic material as well as the biocompability of the natural material. A disadvantage of natural scaffolds is that they are of complex structure and hence manipulation becomes more difficult. They are easily denatured and often require chemical modification which may lead to toxicity [38].

Unmodified natural materials are subject to chemical and enzymatic degradation upon implantation, which decreases the life span of the prosthesis. Whilst active biodegradation can play an important role in tissue remodeling and replacement, this is not favorable for long-term implants, which require slower remodeling to ensure their viability [42]. Therefore, bioscaffolds characteristically require pre-treatment (chemical or physical) to enhance the material's resistance to enzymatic and chemical degradation. For example, many crosslinking techniques to stabilize the collagen-based structure of tissue whilst maintaining its mechanical integrity and natural compliance (such as glutaraldehyde and polyepoxide crosslinking treatments, and dye-mediated photo-oxidation), have been developed to maintain the natural mechanical properties of the scaffold [43].

Processing methods may also serve to sterilize and reduce immunogenicity via decellularization of the tissue. Decellularization techniques include chemical, enzymatic and mechanical means of removing cellular components, leaving a material composed essentially of ECM components. For the most part, these acellular tissues retain natural mechanical properties and promote remodeling of the prosthesis by neovascularization and recellularization by the host [44, 45].

Collagen is a naturally occurring protein that is ubiquitous among mammalian species; therefore, innate commonalities exist in amino acid sequence and epitope structure across species lines [41]. Bovine and porcine type I collagen provide a readily available source of scaffold material for numerous applications and have been shown to be highly compatible with human systems. Collagen can be extracted from tissues such as tendons and ligaments, solubilized, and then reconstituted into fibrils that may be fashioned into a variety of shapes and sizes that mimic body structures. The reconstituted protein is usually stabilized by various methods of cross-linking and must be sterilized prior to surgical use. Certain treatment methods however, significantly alter the mechanical and physical properties of collagen-based materials and may negatively affect the natural physiological processes of cell attachment and proliferation in tissue remodeling. For example, exposure to cross-linking agents such as glutaraldehyde and subsequent sterilization with gamma irradiation can render a collagen-based material biologically inert and incite a foreign body response in the recipient [44].

Collagen provides considerable strength in its natural polymeric state [41]. The necessary and required mechanical and physical properties of tissue engineering products for use in orthopedics, and other body systems, often depends upon chemical manipulation of collagen-based scaffolds. The source of collagen (either purified from animal sources or as an integral component of a more complex extracellular matrix), and its treatment prior to use, are important variables in the design of tissue-engineering devices [41]. Several methods of cross-linking and sterilization can be utilized to alter (i.e. usually decrease) the rate of in vivo degradation or to change the mechanical properties of collagen [43, 46]. These methods include glutaraldehyde treatment, carbodimide treatment, dye-mediated protoxidation, exposure to polyepoxy compounds, and glycerol treatment. Commonly used methods of terminal sterilization include gamma or electron beam irradiation or ethylene oxide treatment [46].

12.4 Porifera Collagen Bioscaffolds for Bone Tissue Engineering

Aside from playing a significant role in the marine ecosystems in which they habitat, the sea sponge, derived from the phylum *Porifera* meaning 'pore bearer', holds both economical and scientific importance. Sea sponges have been utilized as commercial bath sponges since early Greek civilization due to their considerable ability to absorb water, and more recently as potential sources of therapeutic drugs and antibiotic substances [5, 47, 48].

The skeletons of *Porifera* appear to have a number of unique properties that closely mimic bone tissue structure. These properties include its ability to hydrate to a high degree, collagenous of the fiber skeleton, as well as open, interconnected, porous channels created by the fiber network that allows for efficient filtration of nutrients and metabolites [4, 49]. In addition to this, the phylum *Porifera* has a tremendously diverse range of skeletal architecture, with over 8000 extant species currently described, many of which are readily available for biotechnological application [5, 49].

Sea sponges are aquatic, sessile, filter-feeding metazoans [50] that possess a highly mobile population of cells, which maintain an almost protozoan independence. *Porifera* represents the most primitive and simplest metazoan, dating as far as the Precambrian and is considered to be the earliest extant common ancestor of all metazoan [51, 52]. The non-epithelial cells in the sponge are capable of totipotency (differentiating into many cell types such as collagen-secreting cells, spongocytes) to ensure survival of the sponge as a whole [53]. Morphogenetic processes are controlled by extracellular signals and ligands, which interact with their equivalent receptors to trigger signal transduction cascades [54, 55]. With the exception of epithelial cells, they lack anatomically discrete tissues, and therefore do not have reproductive, nervous, muscular, endocrine, circulatory, digestive, excretory, or respiratory systems [5].

Porifera utilize a single layer of flagellated cells (choanocytes) to pump a unidirectional water current through their body. This current travels through the sponge from the inhalant sponge surface pores, known as osti, to exhalant apertures known as oscules [53]. This current is produced by actively beating flagella and the passive movement of environmental currents, which allows for efficient nutrient and gas exchange. The organization of the sponge body is centered around a system of pores, ostia (inhalant pores), canals and chambers in which the low-pressure water currents are conducted. The choanocytes are arranged as a single layered epithelium, termed the choanoderm, which may be continuous or folded, depending on the degree of complexity of the species. The pinacoderm, which can be simple or folded, is the external surface of the sponge and separates the sponge from the environment [50]. It is an epithelial layer that is only one cell deep and is composed of cells called pinacocytes. Between these epithelial layers (the pinacoderm and choanoderm) is an area of connective tissue, the mesophyl, where the skeletal components, organic and inorganic, are secreted and organized [53].

The degree and complexity of the canal systems within the sponge are an important means of Porifera classification, and also provide varying degrees of porosity which may present opportunities for cellular integration when combined with cells for tissue engineering [4]. The simpler systems are referred to as asconoid organization, in which the incurrent and excurrent canals are very short or absent, and the mesophyll is weakly developed. In this system, the osti (inhalant pore) open directly to the main chamber of the sponge body (atrium), which is lined with a continuous layer of choanocytes [50]. The atrium then opens to the external environment via an oscule (exhalant pore). From this system, folding of the pinacoderm and choanoderm produces a syconoid system of organization, of which there are several levels of complexity. The folding of the epithelial layers amplifies the surface area that is in contact with the water current, and forms projections and choanocyte-lined chambers. These chambers open via wide apertures (apopyles) into a single atrium, which then opens to the environment via an oscule. Leuconoid canal systems are the most complex of *Porifera* systems and have great variations. Simply put, this system encompasses further folding of the choanoderm, accompanied by subdivisions of the flagellated surface into additional choanocyte chambers [53]. This organization contains complex canal systems, whereby two or more inhalant canals connect to each choanocyte chamber, and the numerous exhalant canals converge towards the exhalant aperture. In more complex forms of leuconoids, the oscules open into a spacious excurrent cavity (the secondary atrium) with a single large secondary oscule [53]. Leuconoids not only possess an amplified porosity and surface area for nutrient and gas exchange, but additionally contain a denser and more complex mesophyll through which the canal systems travel.

12.4.1 Poriferan Skeletal Structure

The composition and structure of the sponge skeleton largely determines their taxonomic identification within the phylum. The skeletons within the phyla are very diverse as they present numerous fibrous and mineral skeletal combinations and designs, therefore providing opportunities to study various forms as potential bioscaffolds for tissue engineering [4]. The skeleton of *Porifera* can be an exoskeleton, endoskeleton or possibly an intermediate of both, however there are a number of species that do not secrete distinctive skeletal materials [56]. Despite this variation, all of these skeleton types impart shape and rigidity to the interstitial cellular material of the living sponge. Two types of skeletal components occur in sponges: organic collagenous materials, and inorganic materials [50]. The inorganic components of sponge skeletons may be siliceous or calcite, forming spicules. Calcareous spicules are composed chiefly of calcium carbonate in either crystalline calcite or aragonite. Siliceous spicules are amorphous, hydrated silica and have a small quantity of organic matter, called spiculin, which forms an axial fiber [57].

There are two major types of collagen structures found in the extracellular matrix skeleton of sponge. The more common type is dispersed fibrillar collagen, with fibrils approximately 20–30 nm in diameter that disperse throughout the intercellular matrix. It is the only skeletal material found in all sponge species, however the extent to which fibrillar collagen reinforces the sponge extracellular matrix varies greatly within the phylum [50].

The second type of collagen is spongin, a highly compliant material, which may be structured in many forms depending on the species, including fibers, filaments, and spicules. Unlike fibrillar collagen, spongin varies in structure and composition throughout the phylum and in many cases contains embedded or attached silicon spicules or entrapped foreign particles [53]. This variation in structure allows for unique mechanical properties, and gives the sponge survival advantages, for example, the spicule arrangement of marine sponge *Tethya aurantia*, has been demonstrated to have structural-mechanical connections that guard against buckling instability [58]. Spongin fibres, which are larger than collagen fibrils, form complex networks in many sponges and can be divided into primary, secondary and tertiary fibers based on size hierarchy and mechanical strength [5].

In the extracellular matrix of vertebrates, there are three classes of molecules—-collagens and proteoglycans, which are mechanically important molecules, and adhesive glycoproteins, which function to bind cells to the extracellular matrix [59]. The same complex set of molecules found in vertebrate extracellular matrices has also been found to occur in the skeleton of the sponge [60, 61]. These molecules have the same primary structure; however, additional studies are required to understand whether these molecules have the same function throughout the metazoan kingdom. They appear to be involved in cell motility and development in the sponges [50]. The collagenous matrix of sponge is comparable to connective tissues of more complex organisms [53].

Past studies have found the spongin in marine sponge to be analogous to collagen type XIII [4]. Genomic and complementary DNA studies have also identified collagen type IV, which is characteristic for basal laminae, in certain sponge species [62, 63]. The amino acid composition of spongin and collagenous fibrils has been determined by infrared absorption spectra in previous studies, and has been found to be similar to that of vertebrate collagen [50]. The sponge collagen was found to have a very high percentage of glycosylated hydroxylysine, and high levels of aspartic and glutamic acid contents. Glycoproteins and carbohydrate-rich substances are also associated with the spongin collagen, and the adhesion proteins fibronectin and tenascin have also been identified in marine sponges [4]. Sponge collagen however differs from vertebrate collagen in that it possesses seemingly unreducible cross-links between molecules of the tropcollagen element and aromatic compounds, thereby providing greater biological stability [4, 56, 64]. Other unique characteristics found include the presence of arabinose, and the very high insolubility of this collagen [65].

It has recently become evident that the extracellular matrix skeletons of sponge are remarkably complex. The skeletons are not only composed of fibrous molecules, such as collagen, but also corresponding receptors, which are highly similar to those existing in other metazoan phyla, as demonstrated by isolation of cDNAs/genes coding for informative proteins [66]. Signal transduction elements such as receptor tyrosine kinases have been noted, which are representatives of the characteristic metazoan-specific gene families [67, 68]. Sponges possess extracellular adhesion molecules, such as the aggregation factor, which are lectins/galectins [69, 70] that interact with their adhesion receptors composed of scavenger receptor cysteine-rich domains [71]. These, as well as the collagens and responding integrins, form systems that are crucial for specific cell adhesion [68, 72].

Therefore, the extracellular matrix of *Porifera* is not only a matrix that stabilizes the bodily structure of the sponge, but is an active, complex network of molecules that regulate the behavior of the cells and provides the basis for integrated cell communication [51, 70, 73–75].

12.4.2 Classification of Porifera

The general architecture of the skeleton and the chemical nature of the skeletal material have historically been used to differentiate classes and families within the phylum. Groupings within the phylum have also been established by molecular studies analyzing evolutionary novelties of Metazoa [76, 77]. Other morphological characters utilized in classification include shape, reproductive characteristics, distribution and character of the oscula, surface consistency, and pigmentation [53].

At present, four classes within the phylum Porifera are recognized, however only three of the classes have recent species: Calcarea, Hexactinellida, and Demospongiae [5, 55]. The fourth class, Archaeocyatha, was a significant group of sessile marine organisms during the Cambrian Period, however is now presumed to be extinct [5].

The class Calcarea contains sponges that are mostly small in size, and inhabit shallow waters of all seas. They are exclusively marine and are characterized by their skeleton of calcium carbonate spicules and ubiquitous fibrillar collagen. Hexactinellida are marine sponges, however are more common in deeper water. Their skeleton is composed of siliceous spicules, which have a hexactine (six-rayed) structure as well as ubiquitous fibrillar collagen. Demospongiae is the largest class, accounting for approximately 95% of recent species. Representatives can be found in all aquatic environment including fresh/brackish waters, intertidal zones, ocean trenches, and polar to tropical seas. They are characterized by possession of an organic skeleton of collagen which can be dispersed in mesophyl or secreted in the form of large fibres or filaments or spongin. Many species also contain one-rayed (monactine) to four-rayed (tetractine) siliceous spicules in conjunction with, or supplementary to, the collagenous framework. Demospongiae is a structurally diverse class, including forms which possess only a fibrillar dispersed collagenous skeleton, forms with fibrillar collagen in addition to a fibrous collagenous skeleton, forms with fibrillar collagen, spongin fires and silicon spicules, and forms where a basal calcareous skeleton is present in addition to the collagen and spicule skeleton [5, 53].

12.4.3 Analysis of Porifera for Bone Tissue Engineering

Porifera display a structure which mimics the cancellous architecture of bone tissue. The complex canal system within sponges creates a porous environment that is ideal for cellular integration when combined with cells for tissue engineering. The Western Australia coast houses a large and diverse range of marine sponges including rare species unique to the area [78]. Over the last 20 years, we have studied the potential of marine sponges as a bioscaffold to promote osteogenesis in bone tissue engineering.

The sponges collected from the Fremantle coast of Western Australian are a member of the sponge family Callyspongiidae (belonging to the order Haplosleridia). Species 1 is an erect, branched, tubular sponge with a large oscule (exhalent aperture) at the end of each tubular projection (Fig. 12.1a). The surface of the sponge is covered with extruding fibers forming conules which gives the surface a bumpy appearance. Species 2 is an irregularly shaped, cushion-like, encrusting sponge with oscules scattered at regular intervals over its surface (Fig. 12.1b). Species 3 has complex highly branching, tubular morphology, with a smooth surface (Fig. 12.1c). Each irregularly sized tube ends in a knob with an oscule. Species 4 is an irregularly shaped cushion-like, thickly encrusting sponge (Fig. 12.1d). Small irregularly shaped projections extend from the central mass, each ending in a knob with an oscule. Species 5 is composed of vase-shaped, erect projections each with a terminal oscule (Fig. 12.1e). The surface of this sponge is highly conulose, with tufts of emergent fibers providing a furry appearance on the sponge surface.

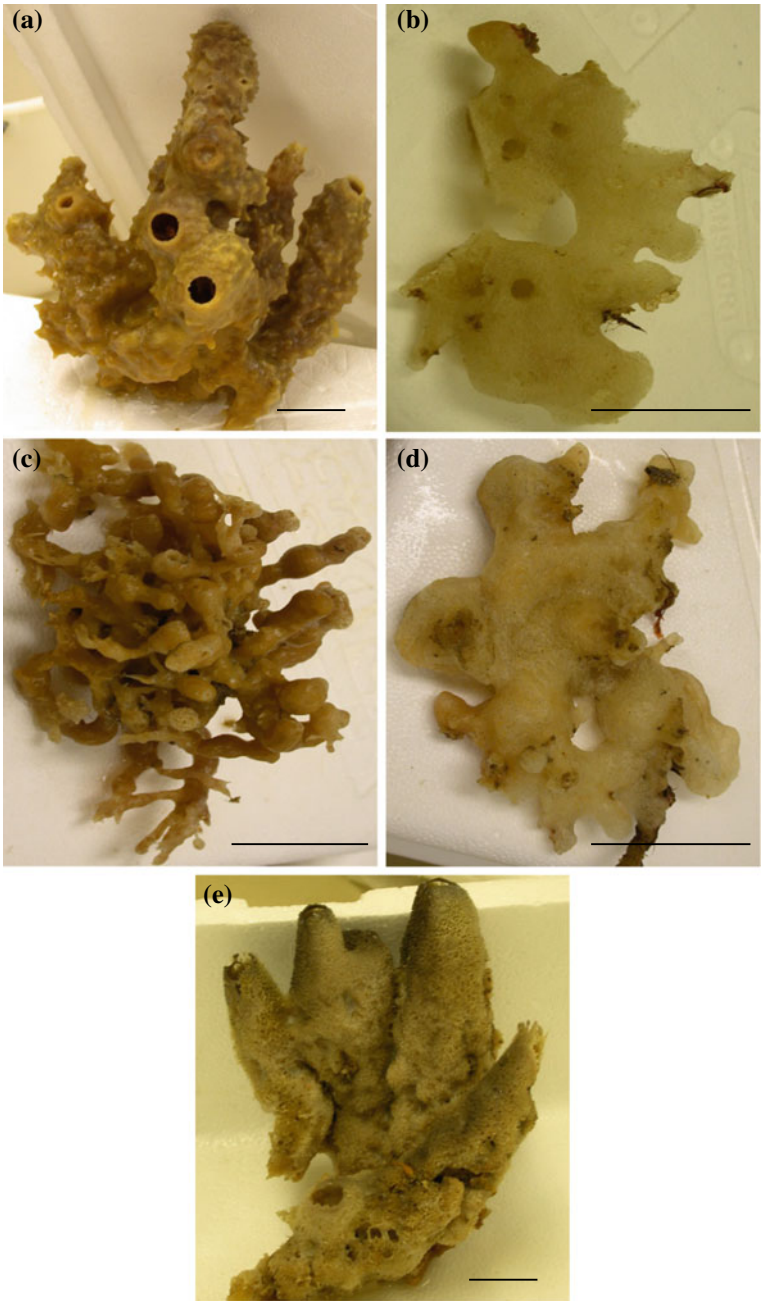

Fig. 12.1 Photographs of the sponge samples as collected. **a** ***Species 1*** has an erect, branched, tubular morphology; **b** ***Species 2*** is an irregularly shaped cushion-like sponge; **c** ***Species 3*** has a highly branching tubular morphology, and smooth surface; **d** ***Species 4*** is an irregularly shaped cushion-like sponge; **e** ***Species 5*** has vase-shaped erect projections and a conulose surface (Scale bar = 5 cm)

The skeleton of Species 1, 2 and 3 are composed of regularly interconnected spongin fibers forming the choanosomal (mesophylic) skeleton (Fig. 12.2a, c, e). The primary, secondary and occasional tertiary fibers (Fig. 12.2d) form rectangularly meshed networks, whereby the primary fibres are either ramified (branched) to form secondary fibers and tertiary fibers (Species 2 and 3), or are non-ramified, parallel, and connected by short parallel secondary fibers (Species 1). These properties are characteristic of the genus Callyspongia (Family Callyspongiidae, Suborder Haplosclerina, Order Haplosclerida). This genus is also known for its two-dimensional ectosomal (external) skeleton of primary, secondary and occasional tertiary spongin fibers (Fig. 12.2f), and the presence of oxeate (tapered with two pointed ends) and strongylate (two dissimilar ends, one pointed and one rounded) siliceous spicules (Fig. 12.2b, d). The parallel primary fibers of Species 1 range from 200 to 300 μm in diameter, and connecting secondary fibers measure approximately 100 μm diameter (Fig. 12.2a). The interconnected spongin fibers create pores throughout the sponge skeleton. The majority of pores within the skeleton of Species 1 range from 100 to 300 μm. Siliceous spicules were attached to the fibers of Species 1 before it was processed (Fig. 12.2b) however were not found attached to the external fiber surface after processing (Fig. 12.2a). The spongin fibers of Species 2 range from 30 to 50 μm in diameter, and create pores ranging from 100 to 300 μm in diameter. The smooth fibers of Species 2 had occasional siliceous spicules attached to or embedded within the fiber (Fig. 12.2d). These spicules are strongylate in shape, 20–40 μm in length aligning longitudinally along the fiber axis.

The fibers comprising the choanosomal (internal) skeleton of Species 3 range from 40 to 60 μm in diameter, and create pores of 250–600 μm in diameter (Fig. 12.2e). The ectosomal (exterior) skeleton of the sponge branching off primary fibers of the choanosomal skeleton, is composed of a dense, two-dimensional matrix of primary, secondary and tertiary fibers (30, 20 and 10 μm in diameter respectively) (Fig. 12.2f). Pores formed on the two-dimensional ectosomal skeleton range from 50 to 80 μm in diameter. The fibers within the whole skeleton have smooth surfaces with occasional attached or embedded oxeate and strongylate spicules (about 50 μm in length). No cellular debris was seen on the skeleton of either Species 1, 2 or 3.

Species 4 appears to be a member of the family Chalinidae, (Suborder Haplosclerina, Order Haplosleridia) due to its reticulated skeleton of connected spongin fibers, the thickly encrusting, cushiony growth form, and the characteristically abundant oxeate and strongyle siliceous spicules. The sponge could not be classified to genus level due to insufficient information about this sponge; however, it shows signs of belonging to the genus Haliclona (smooth surface of ectosomal skeleton and characteristic oscular mounds). The irregularly spaced, extensively ramified, smooth spongin fibers are approximately 100 μm in diameter (Fig. 12.2g). Pores vary greatly in size and shape however, most are round, 100–250 μm in diameter. This sponge has

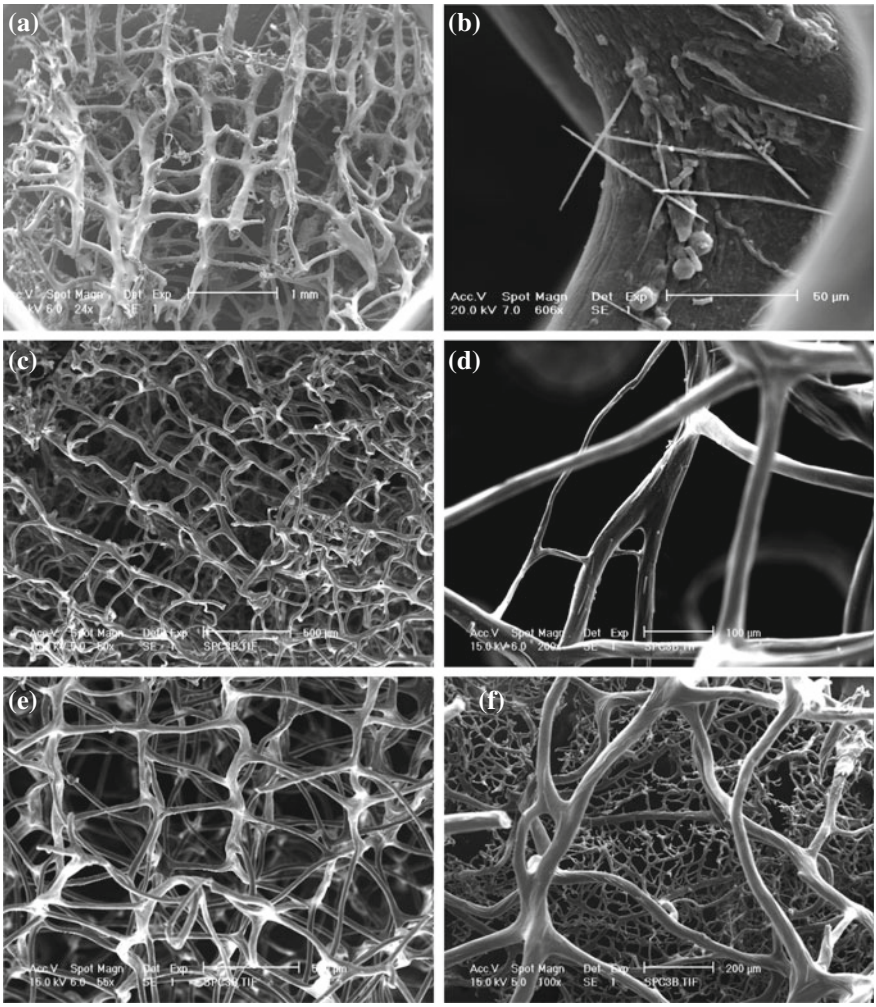

Fig. 12.2 SEM micrographs of sponge skeletons. ***Species 1*** (**a**) (processed) showing a regular spaced fibre network, and (**b**) (unprocessed) portraying spicules attached to fiber surface; ***Species 2*** (**c**) (processed) at low magnification showing regular arrangement of fibers, and (**d**) at high magnification showing tertiary fibers and spicules; ***Species 3*** (processed), (**e**) at low magnification showing the regular fiber network, and (**f**) at high magnification displaying the ectosomal skeleton branching off the choanosomal fiber network, and occasional spicules; ***Species 4*** (processed), (**g**) at low magnification showing the irregular mass of fibers, and (**h**) at high magnification showing spicules attached to and embedded within the fibers; ***Species 5*** (processed), (**i**) at low magnification showing irregularly arranged fibers with some cellular debris (webbing) between the fibers, and (**j**) at high magnification showing the rough surface of the fibers

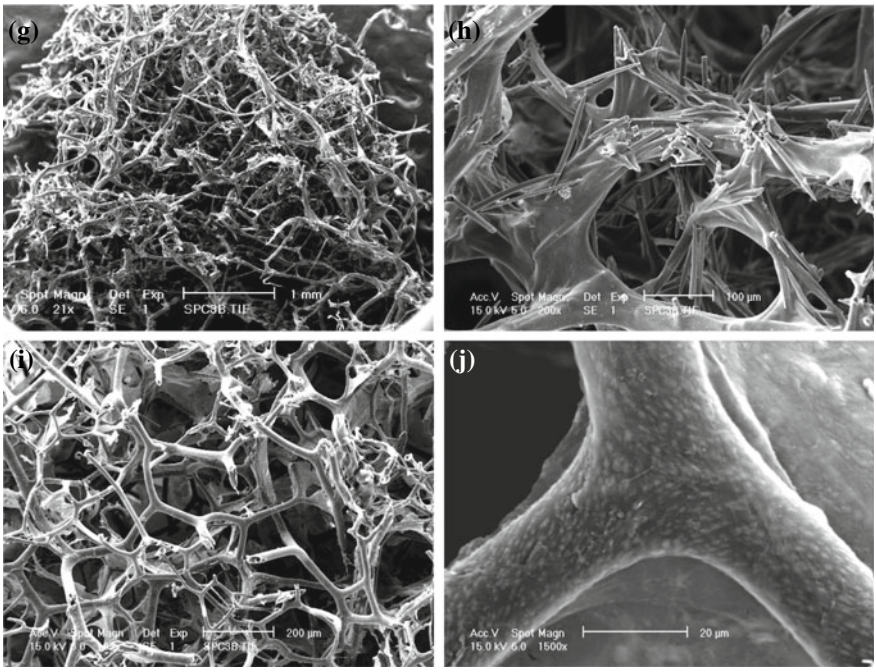

Fig. 12.2 (continued)

an abundance of siliceous spicules both embedded within, and attached to, the fiber skeleton (Fig. 12.2h). The spicules appear to cover approximately 20% of the fiber network and are imbedded within and attached to the fibres with random orientation (Fig. 12.2h). The spicules range from 40 to 150 µm in length and are straight oxeate or strongylate in morphology.

Species 5 has features characteristic of the genus Hippospongia (Family Spongiidae, Order Dictyoceratida) having a dense fiber skeleton dominated by a reticulum of irregular secondary fibres (Fig. 12.2i) and a lacunose (highly cavenous) structure. The secondary fibers of the skeleton are 25 µm in diameter, and the tertiary fibers 15 µm, however no primary fibers are present (Fig. 12.2i). The matrix is irregular and ramified, with no visible spicules. Pores dimensions are between 50 and 200 µm in diameter (Fig. 12.2i). On closer examination, the fibers are seen to have rough surfaces (Fig. 12.2j). In the processed sponge there appear to be membranes stretching between some of the fibers, covering pores within the skeleton (Fig. 12.2i, j).

12.5 Characterization of Osteoblast-Seeded Sponge Skeletons In Vitro

To study the effectiveness of sea sponges on induction of bone formation, we have grown primary osteoblasts from mice on sea sponges in osteoblast differentiation medium containing a cocktail of 100 nM dexamethasone, 10 mM β-glycerophosphate, and 50 μg/ml ascorbic acid-2-phosphate (SIGMA-ALDRICH, St. Louis, MO, USA) to a final concentration of 5×10^5 cells/ml. Pre-soaked pieces of sponge were placed separately in the wells of 24-well plates, and 1 ml of cell suspension was slowly added to each well. Cells were cultured at 37 °C, 5% CO_2 for 10–12 h (to allow cellular adherence). Seeded constructs were then given a further ml of differentiation medium. Medium was changed every 2–3 days. Sponge-cell constructs were cultured for 4, 7, 14 and 21 days. At 4 and 14 days, samples were fixed and subjected to confocal microscopy examination. At 7, 14 and 21 days RNA was extracted from samples and cDNA used for real-time PCR analysis to measure expression of osteoblastic gene markers.

12.5.1 Short Term Osteoblast Culture on Porifera

After four days culture, cells attached to the spongin fibers of all sponge species, however cell attachment appears tenuous (Figs. 12.3 and 12.4). In most cases attached osteoblasts are individual and of spindle-shaped morphology (Fig. 12.3). Osteoblasts have extended congruent with the matrix fibers, with bi- or tri-polar attachment in most cases (Fig. 12.3). Cells grown on Species 2 can be seen to bridge interconnected spongin fibers of the choanosomal (Fig. 12.3d) and ectosomal skeletons (Fig. 12.4d). Cell seeded onto the skeleton of Species 1 attached with and without serum-supplemented medium (Figs. 12.3a, b and 12.4a, b). However, cells appear apoptotic on serum-free medium due to very tenuous attachment and spherical morphology (Fig. 12.3b). Cells stained with F-actin fluoresce red when examined by confocal fluorescence microscopy, and Hoechst bis-benzimide staining caused the cell nuclei to fluoresce blue/purple (Fig. 12.4). The spongin fibres of the sponge skeletons autofluoresce green. Figure 12.4d shows the ectosomal skeleton of Species 3 where the osteoblasts can be seen bridging the pores. The skeleton of Species 5 appears to have remnant cellular debris incorporated in its skeleton, which cells can be seen spreading on to (Fig. 12.4f, g).

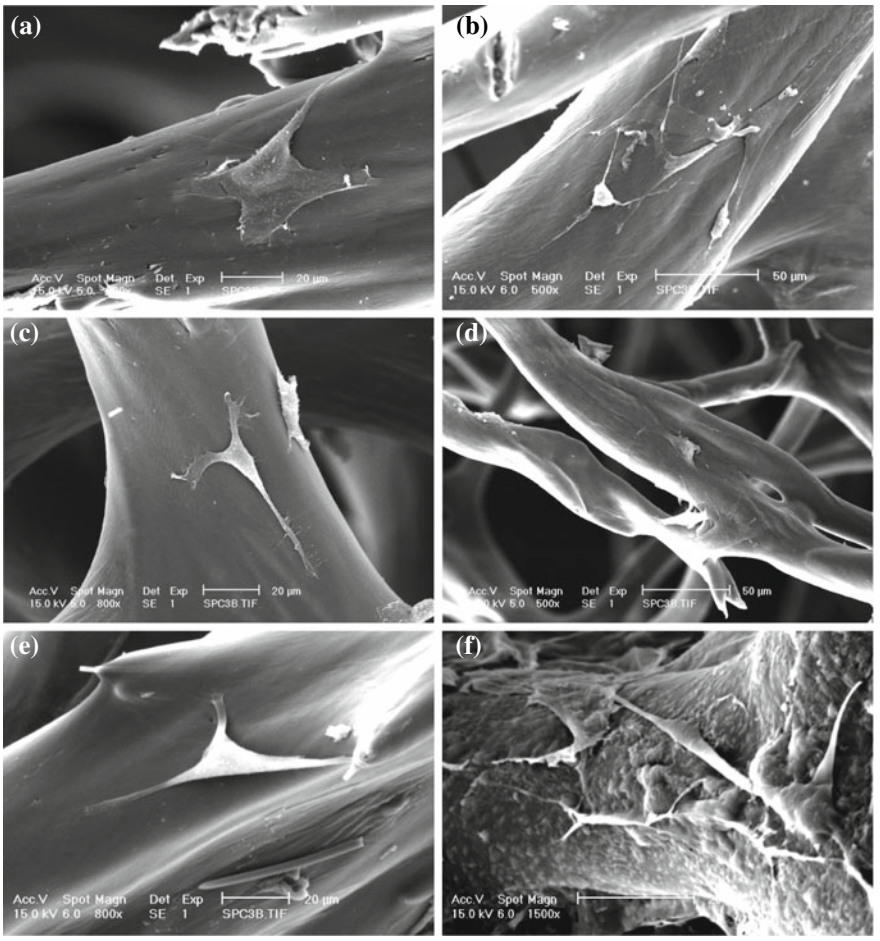

Fig. 12.3 SEM micrographs of osteoblast-sponge constructs after 4 days *in vitro* culture. **a** A spindle-shaped cell attached to the surface of a fiber of ***Species 1***; **b** Cells attached to the skeleton of ***Species 1*** which had been incubated with serum-free medium; **c** A cell on the surface of a fiber of ***Species 2***, showing spindle-like morphology and tri-polar attachment; **d** Cells bridging between connecting fibers of ***Species 3***; **e** The tenuous tri-polar attachment of a spindle-shaped cell on ***Species 4***; **f** Cells attached to the rough surface of a fiber of ***Species 5***

12.5.2 Long Term Osteoblast Culture on Porifera

Seven days after cell seeding of the constructs there was a significant increase in cell proliferation on all sponge skeletons (Figs. 12.5 and 12.6). Alkaline phosphatase activity is expressed in some cells on all scaffolds (Fig. 12.6b, d, f, h, i). A cluster of globular shaped cells can be seen spreading over the surface of two fibers of Species 1 (Fig. 12.5a). Osteoblasts can also be seen forming a layer over the fibers of Species

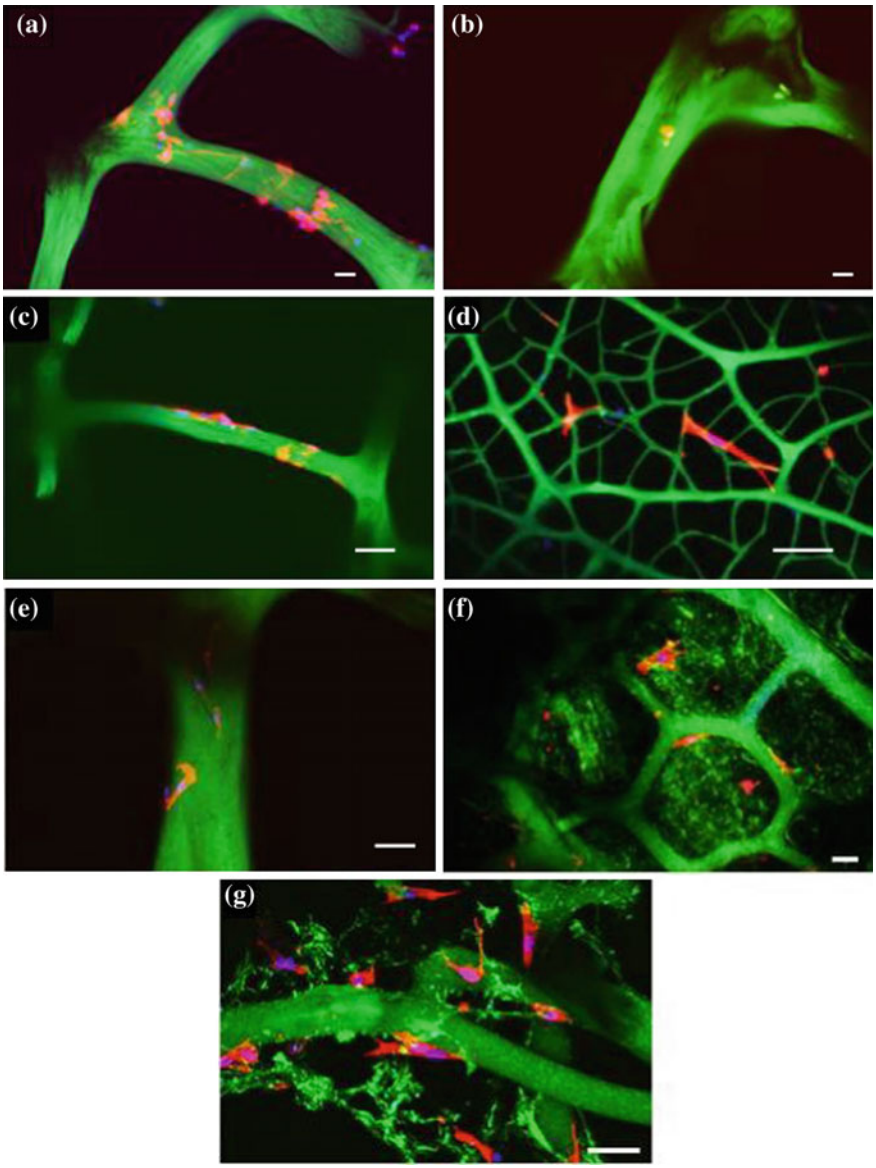

Fig. 12.4 Fluorescent confocal micrographs of sponge-cell constructs after 4 days culture. Cells were F-actin immunostained which caused cells to fluoresce red. Constructs were also stained with Heochst bis-benzimide to show the cell nucleus (blue/purple fluorescence). The spongin fibres possess autofluorescence (green). **a** Attachment of cells to the fibers of ***Species 1***; **b** ***Species 1*** in serum-free medium, cell appears apoptotic; **c** Attachment of cells to a fiber of ***Species 2***; **d** Cells bridging pores of the ectosomal skeletal fibers of ***Species 3***; **e** Attachment of a cell on a fiber of ***Species 4***; **f** Cells within the skeleton of ***Species 5*** at low magnification. Debris of some kind can also be seen; **g** Cellular attachment on the fibers and debris within the skeleton of ***Species 5***, at high magnification (Scale bar = 20 μm)

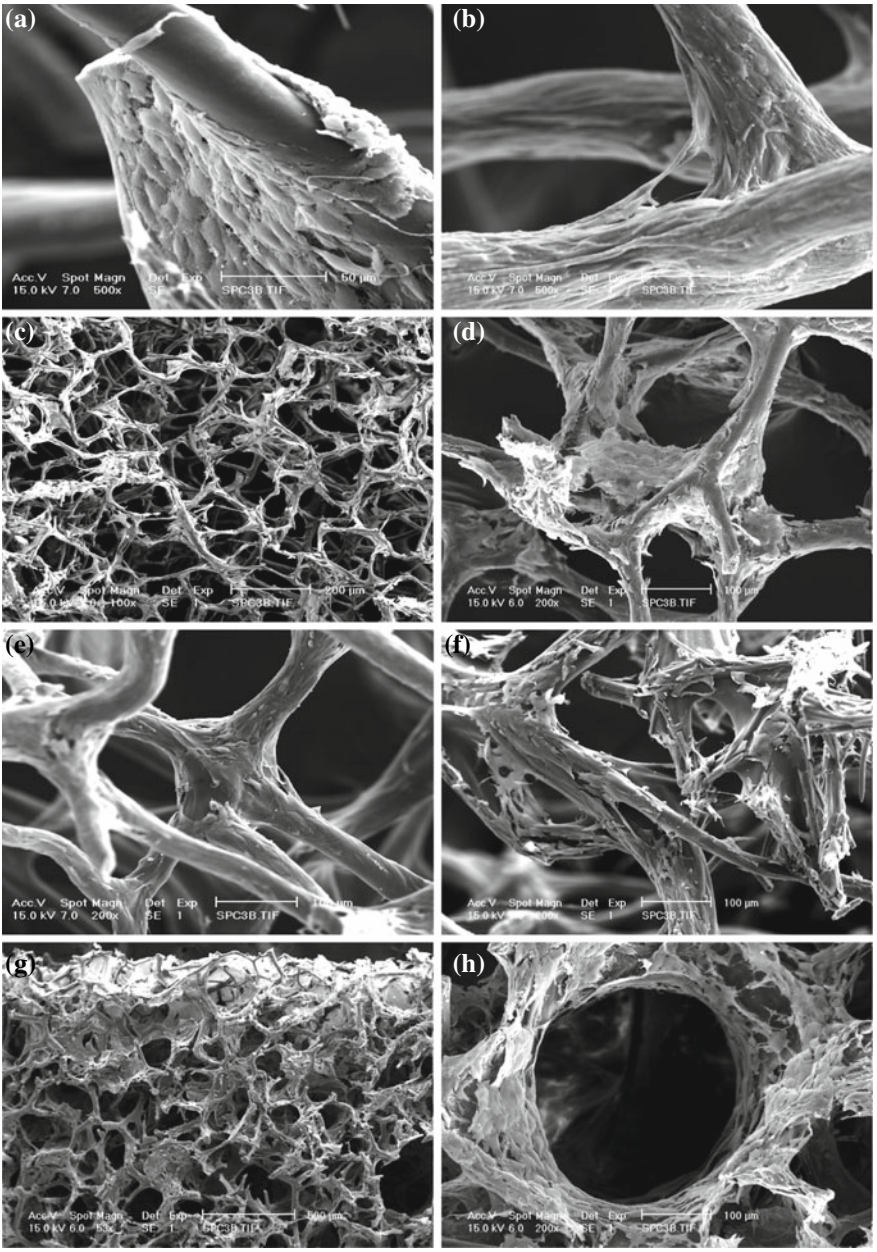

Fig. 12.5 SEM micrographs of sponge-cell constructs after 7 days culture. **a** and **b** ***Species 1*** showing cells with globular and spindle-like morphology; ***Species 2*** at low (**c**) and high (**d**) magnification showing heterologous cell coverage; **e** ***Species 3*** showing cells forming layers over the fibres of the sponge skeleton; **f** ***Species 4*** showing sporadic cell growth over fibers and spicules; ***Species 5*** at low (**g**) and high (**h**) magnification, showing limited filling-in of large pores by cells

1, and forming a bridge between two perpendicular connected fibers (Figs. 12.5b and 12.6a). Cell coverage of the sponge skeleton is heterologous (not uniform) on the skeleton on Species 2 (Figs. 12.5c). At high magnification a combination of cell shapes, mostly spindle-like can be seen growing congruent with the matrix fibers (Fig. 12.5d). There is some evidence of spherical osteoblasts in clusters. Spindle-shaped cells have grown along the longitudinal axis, and covering the interconnecting nodes, of fibers in the choanosomal skeleton of Species 3 (Figs. 12.5e and 12.6e).

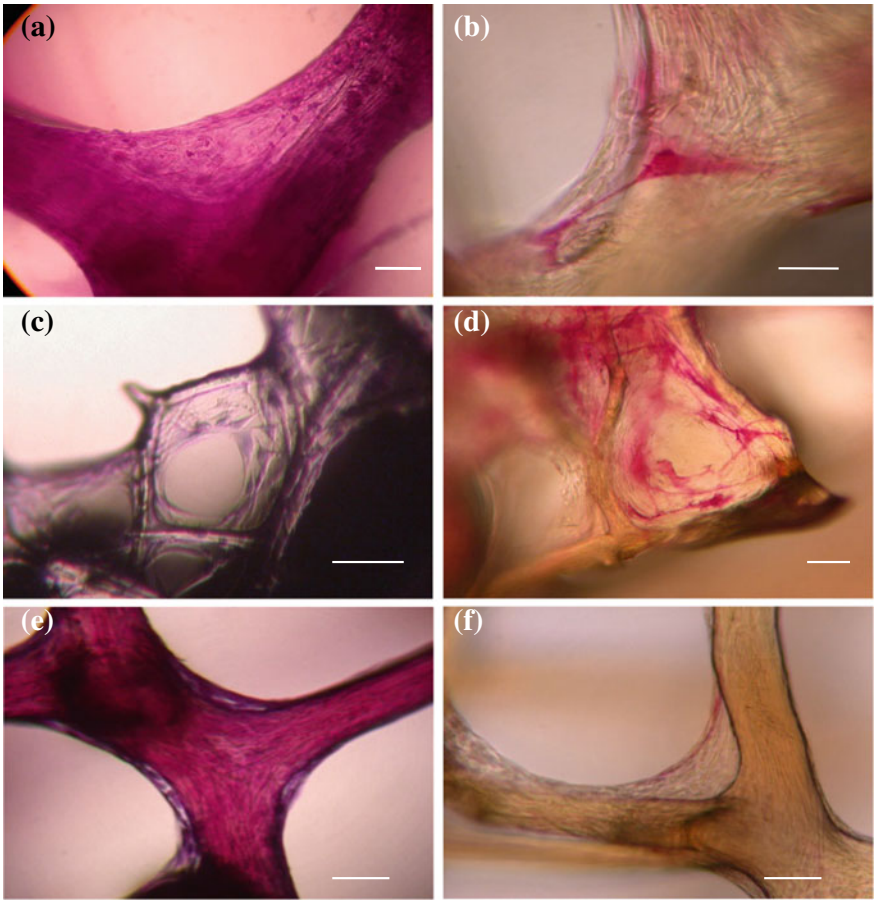

Fig. 12.6 Light micrographs of sponge-cell constructs after 7 days *in vitro* culture. **a** ***Species 1*** stained with Haemotoxylin and Eosin (H+E) showing cellular bridging between connecting fibers, and **b** stained for Alkaline phosphatase (ALP) activity, showing the osteoblastic phenotype of some cells (red); **c** ***Species 2*** stained with H+E showing infiltration of a pore, and **d** stained for ALP activity; **e** ***Species 3*** stained with H+E showing a layer of cells over the fibres, and **f** stained for ALP showing only slight activity; **g** ***Species 4*** stained with H+E showing cell bridge interconnecting fibres, and **h** stained for ALP showing limited activity; **i** ***Species 5*** stained with H+E showing cell coverage of pores, and **j** stained for ALP showing significant activity (Scale bar = 40 μm)

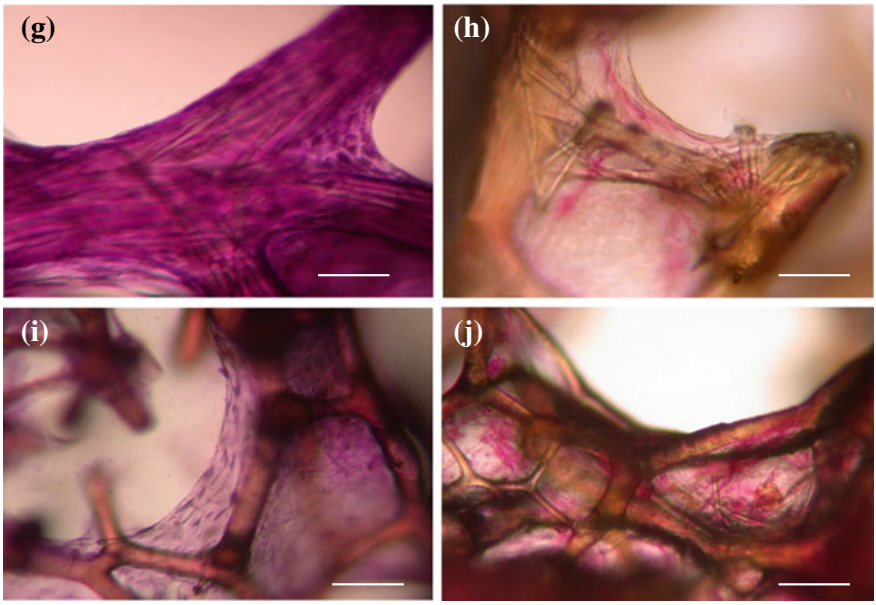

Fig. 12.6 (continued)

Cells with spindle-like morphology can also be seen to elongate longitudinally along the fibers of Species 4 (Fig. 12.5f), and bridging fibers (Fig. 12.6g). Cell growth is sporadic and heterologous; and attachment to spicules is minimal. Under low magnification of a transverse section of a Species 5 cell-sponge construct (Fig. 12.5g) display heterologous cell coverage, with limited cellular infiltration deep within the matrix. Large pores (>200 μm diameter) have not been bridged or filled in by cells, however globular shaped cells can be seen infiltrating the large pores, growing around their circumference (Fig. 12.5h).

After 14 days of in vitro culture, there was an increase in cell proliferation over the extracellular matrix of all species of sponge skeletons. The majority of proliferation shown on the skeletal fibers of Species 1 is by cells forming mats over the fibers and bridging small pores (Figs. 12.7a, b, 12.8a and 12.9a). Extensive proliferation was observed over the fiber skeleton of Species 2 (Figs. 12.7c, d, 12.8b and 12.9b). Heterologous spreading of spindle-shaped cells occurred over the scaffold with mats formed over many pores. ALP activity for Species 1 and 2 is high (Fig. 12.8b and d). Species 3 was found to have extensive proliferation and coverage of its ectosomal skeleton where cells adopted a more globular morphology (Figs. 12.7e and 12.9c) with high ALP activity (Fig. 12.8f).

However, cell coverage was limited to surrounding the fibers of the choanosomal skeleton, without much infiltration of pores (Fig. 12.8e). Figure 12.8f shows the moderate coverage of Species 4 where cells covered the fibers of the sponge and started to adopt a more globular morphology. There appears to be limited cell growth

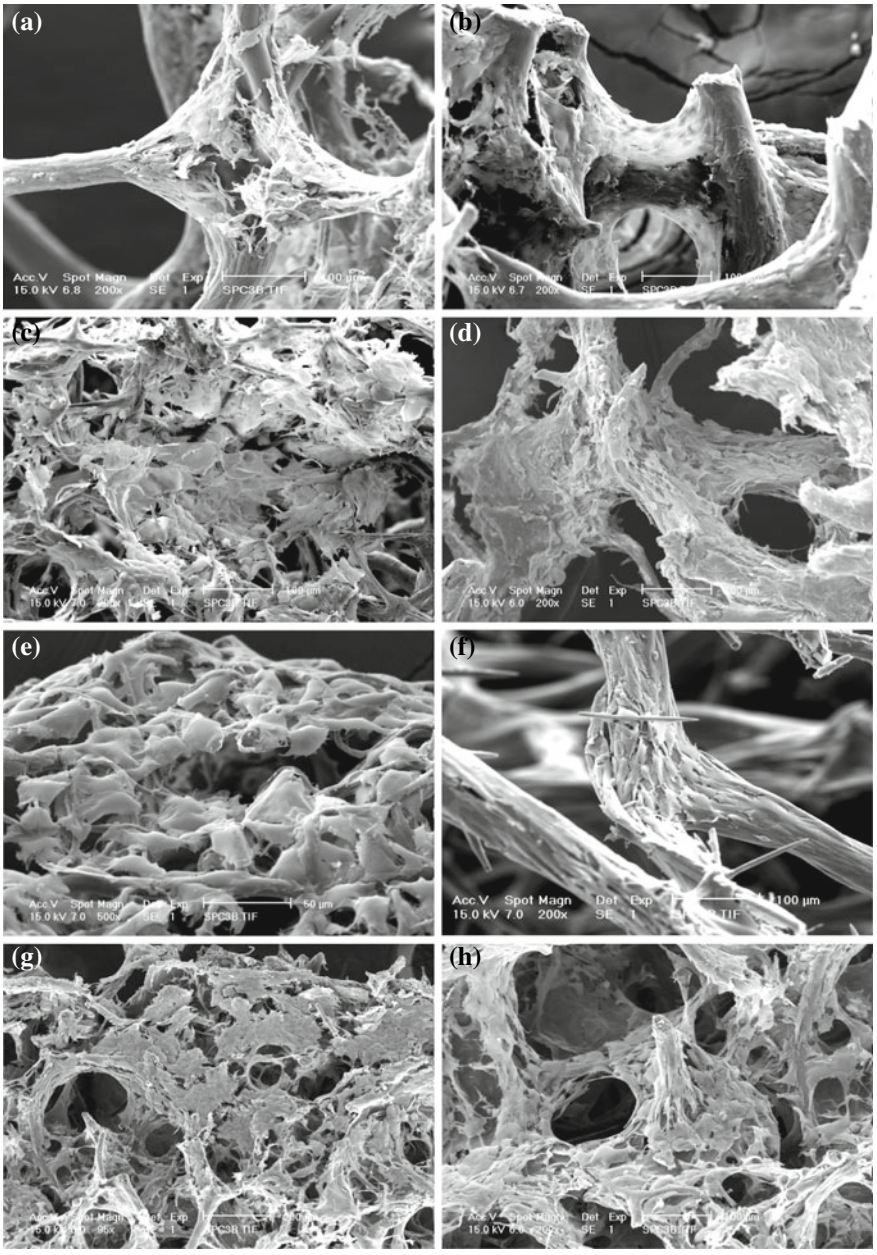

Fig. 12.7 SEM micrographs of sponge-cell constructs after 14 days *in vitro* culture. **a** and **b** ***Species 1*** showing cellular coverage of fibers and bridging of pores; **c** and **d** ***Species 2*** portraying the extensive proliferation of spindle-shaped cells; **e** ***Species 3*** showing globular shaped cells aggregating over the ectosomal skeleton; **f** ***Species 4*** reveals that cells start adopting more globular morphology, however limited coverage of spicules is seen; **g** and **h** ***Species 5*** displays the infiltration of pores and the formation of mats

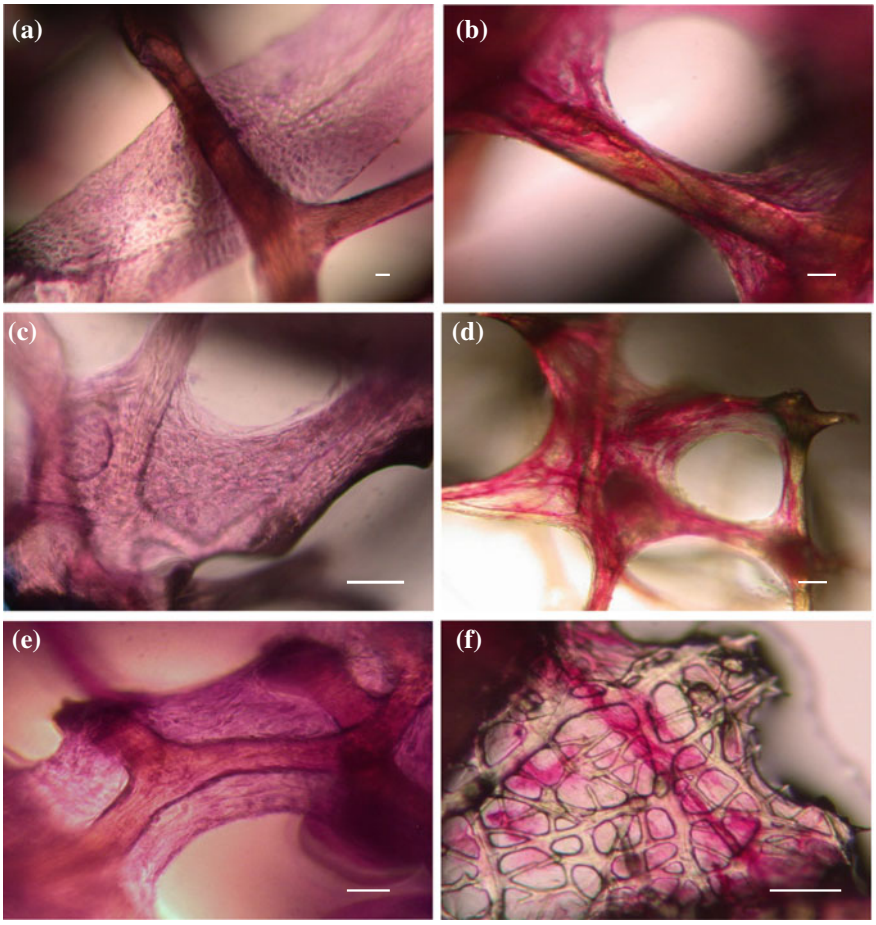

Fig. 12.8 Light micrographs of sponge-cell constructs after 14 days *in vitro* culture. **a** ***Species 1*** stained with Haemotoxylin and Eosin (H+E), and **b** stained for Alkaline phosphatase (ALP) activity, which is highly active; **c** ***Species 2*** stained with H+E, and **d** stained for ALP which is highly active; **e** ***Species 3*** stained with H+E, and **f** the ectosomal skeleton stained for ALP activity; **g** ***Species 4*** stained with H+E; and **h** stained for ALP; **i** ***Species 5*** stained with H+E, and **j** stained for ALP (Scale bar = 50 μm)

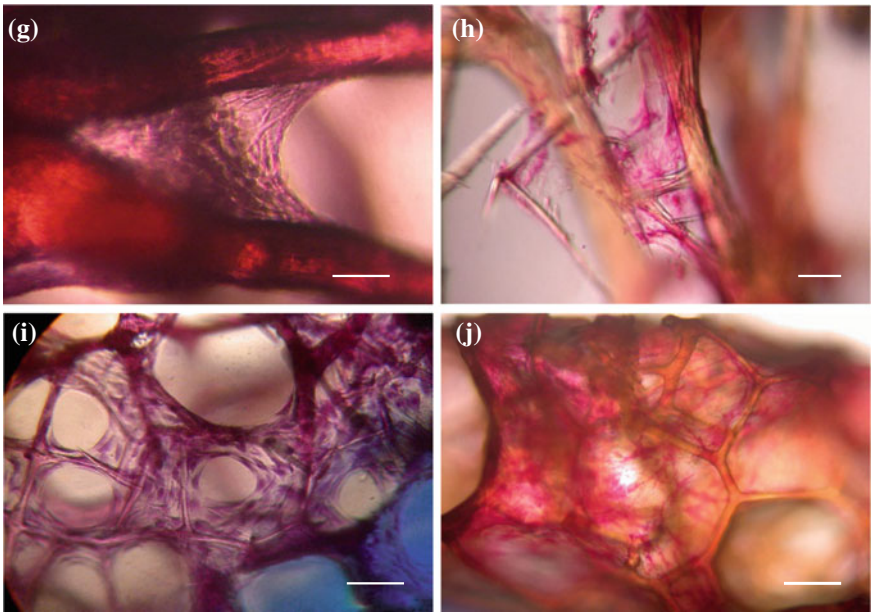

Fig. 12.8 (continued)

on spicules. Cells appear to extend over the silicon spicules to cover the spongin fibers however do not form aggregations on the spicules as they do on the fibers. Cells form bridges between connecting fibers and are beginning to fill small pores of Species 4 (Fig. 12.8g and 12.9d). The surface of Species 5 appears to be approximately 40% covered with cellular aggregations starting to form mats, however individual cells can still be seen (Fig. 12.7g). Remnants of sponge membranes (webbing) can still be seen in some areas, with little evidence of cell growth. Cells of globular/ovoid morphology have formed bridges between fibers and across pores of Species 5 viewed under high magnification (Fig. 12.7h), however no matting has occurred in this area.

Species 2 and 5 were found to be almost completely encapsulated by cells after 21 days in vitro culture (Fig. 12.10c, j). However, transverse sections of Species 5 showed only the surface to be covered with osteoblastic cellular matting, as confirmed by ALP staining (Fig. 12.11j). Comparably, Fig. 12.10i also showed that cells did not infiltrate more than 800 μm into the construct. Species 1 showed almost full cellular coverage of the construct within 21 days culture; however, cells did not completely bridge pores over 500 μm in diameter (Figs. 12.10a and 12.11a). The ectosomal skeleton of Species 7 was completely matted over with a layer of osteoblasts within the 21 days culture (Figs. 12.10e, f, and 12.11e), however penetration of pores and

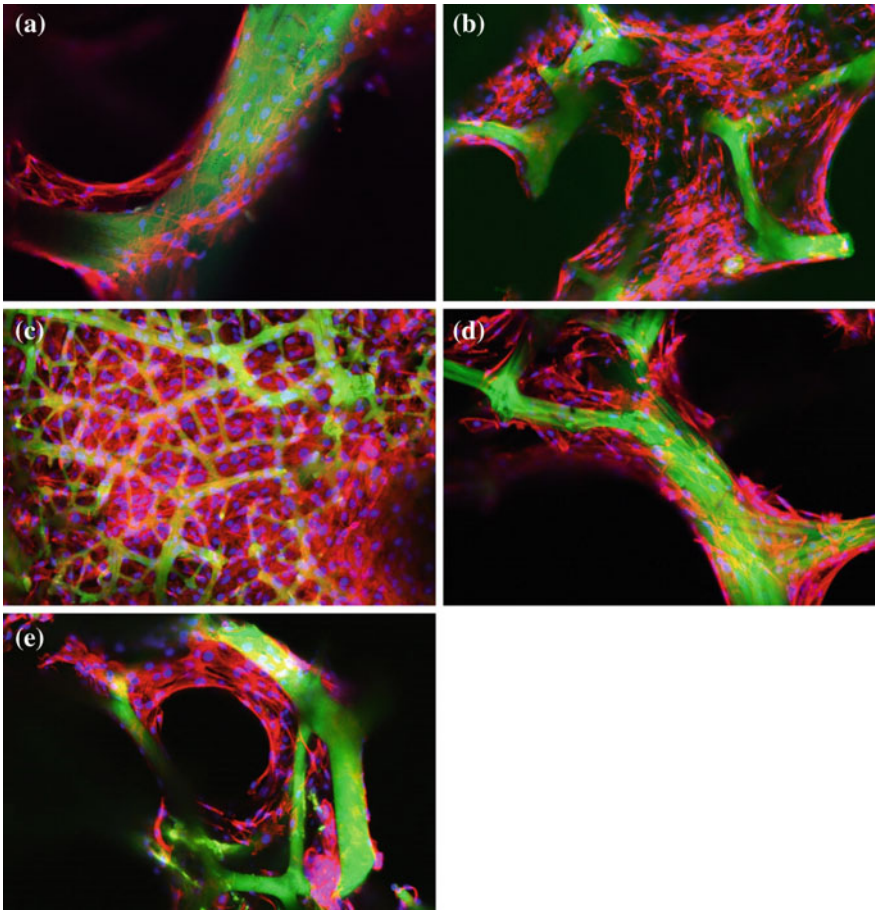

Fig. 12.9 Confocal microscopy showing F-actin fluorescent staining of cells (red) on sponge skeletons (green autofluorescence) after 14 days culture. **a** Cells have formed thin layers over the fibers of ***Species 1***; **b** infiltration of pores in ***Species 2***; **c** Cells form a mat over the ectosomal skeleton of ***Species 3***; **d** Cells form thin layers over the fibers of ***Species 4***; **e** Infiltration of a pore of the skeleton of ***Species 5*** (Scale bar = 20 μm)

coverage of fibers within the choanosomal skeleton was limited and with negligible osteoblastic phenotype (Fig. 12.11f). SEM images (Figs. 12.10g, h) of Species 4 show very moderate cell coverage. At low magnification, the scaffold can be seen to be porous and almost devoid of coverage (Fig. 12.10g). At high magnification, cells can be seen heterologously and sporadically covering spongin fibers (Figs. 12.10h and 12.11g), with confirmed osteoblastic phenotype (Fig. 12.11h). No degradation of sponge scaffolds was observed during the 21 days of in vitro culture.

In summary, primary osteoblasts were found to attach, infiltrate and grow on the devitalized skeletons of five different sponge species of *Porifera*. Osteoblastic

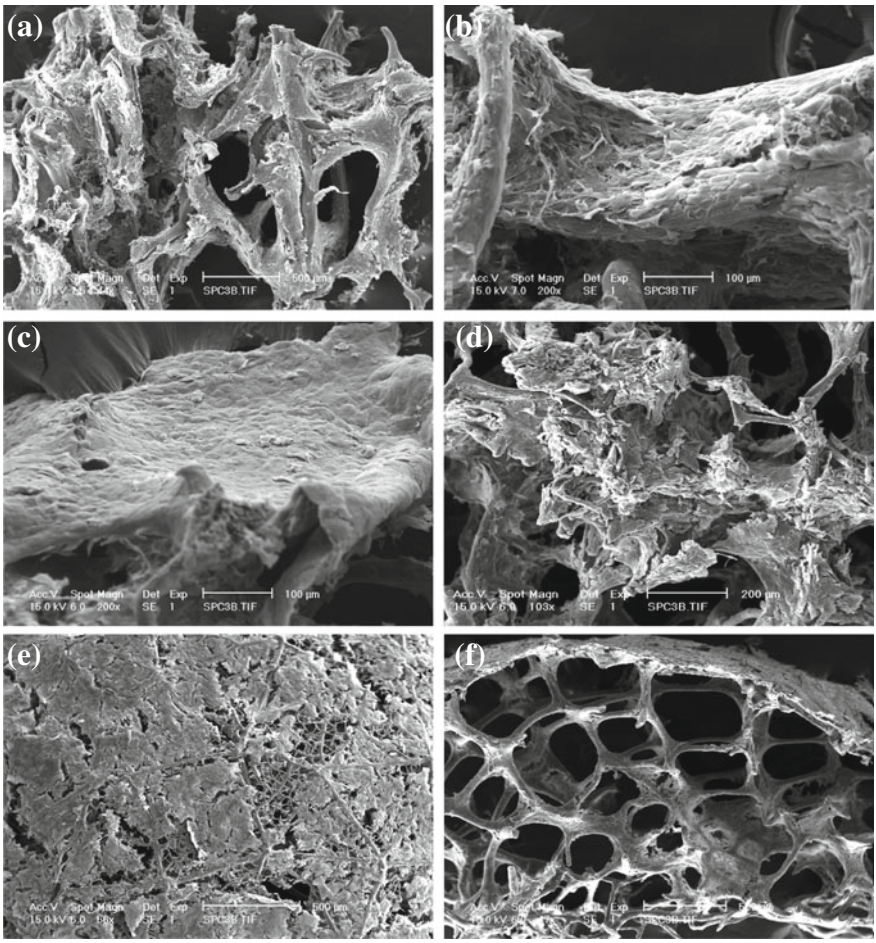

Fig. 12.10 SEM micrographs of sponge-cell constructs after 21 days. ***Species 1*** at low magnification (**a**) and high magnification (**b**) showing cell coverage; ***Species 2*** at low magnification (**c**) and high magnification (**d**) showing encapsulation of the matrix; ***Species 3*** showing matting over the ectosomal skeleton (**e**), and (**f**) showing limited cell coverage of the choanosomal matrix; ***Species 4*** at low magnification (**g**) and at high magnification (**h**) showing limited cell coverage; ***Species 5*** at low magnification (**i**) and at high magnification (**j**) showing matting of the surface of the construct

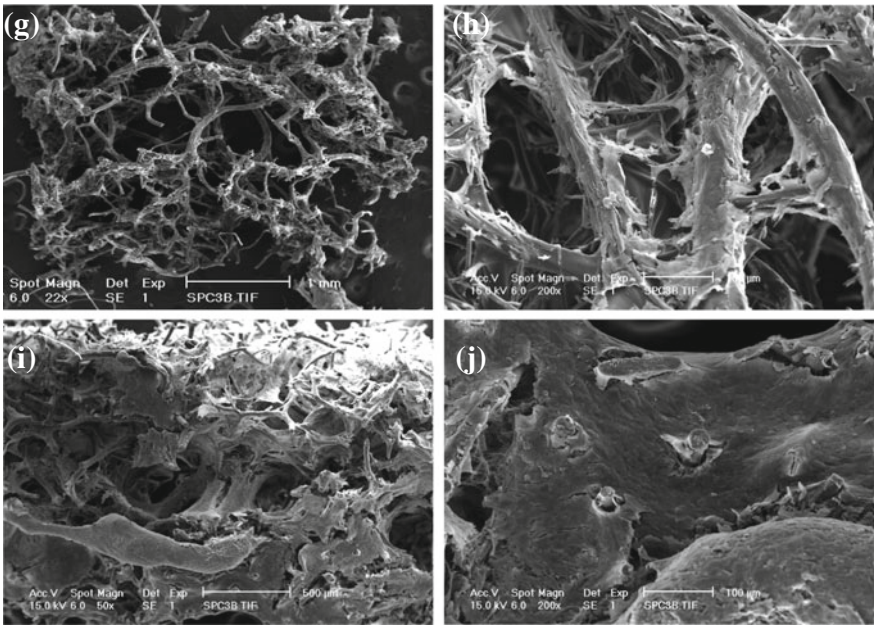

Fig. 12.10 (continued)

phenotype was confirmed by observed alkaline phosphatase activity. Proliferation continued over a 21-day in vitro culture period, whereby cells infiltrated pores up to 600 μm in diameter. Osteoblasts were first found align congruent with the spongin fibers of the sponge, then proceeded to aggregate around interconnecting nodes of the matrices. Cells were found to completely bridge many pores created by the spongin fiber network. Total cellular encapsulation of the sponge skeleton was observed in some species after 21 days culture.

12.6 Biocompatibility of *Porifera* for Bone Tissue Engineering

One of the major criteria that enable *Porifera* to be used as implantable materials for bone tissue engineering is their biocompatibility. To examine if *Porifera* scaffold is biocompatible for in vivo implant, we have processed the sea sponges to free the sponge skeleton off cellular debris (Zheng, MH. Australia Provision Patent No 2008901451) and were γ-irradiated and stored in sterile conditions at −4 °C prior for

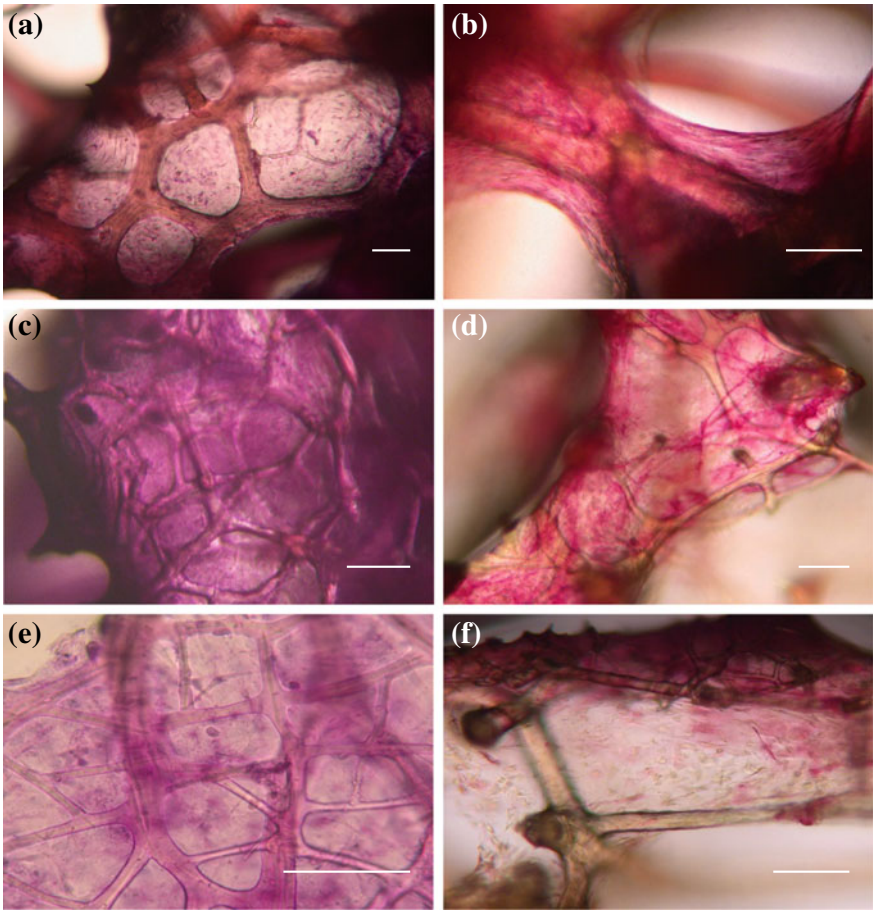

Fig. 12.11 Light micrographs of sponge-cell constructs after 21 days *in vitro* culture, showing infiltration of pores and osteoblastic phenotype of cells. **a** ***Species 1*** stained with Haemotoxylin and Eosin (H+E), and **b** stained for Alkaline phosphatase (ALP) activity; **c** ***Species 2*** stained with H+E, and **d** stained for ALP activity; **e** ***Species 3*** showing the ectosomal skeleton stained with H+E, and **f** stained for ALP, showing the limited ALP activity of cells on the choanosomal skeleton as compared to that of the cells on the ectosomal skeleton; **g** ***Species 4*** stained with H+E, and **h** stained for ALP activity; **i** ***Species 5*** stained with H+E showing encapsulation of a portions of the construct, and **j** a transverse section of the sponge-cell construct stained for ALP activity, showing limited penetration of osteoblastic cells (Scale bar = 100 μm)

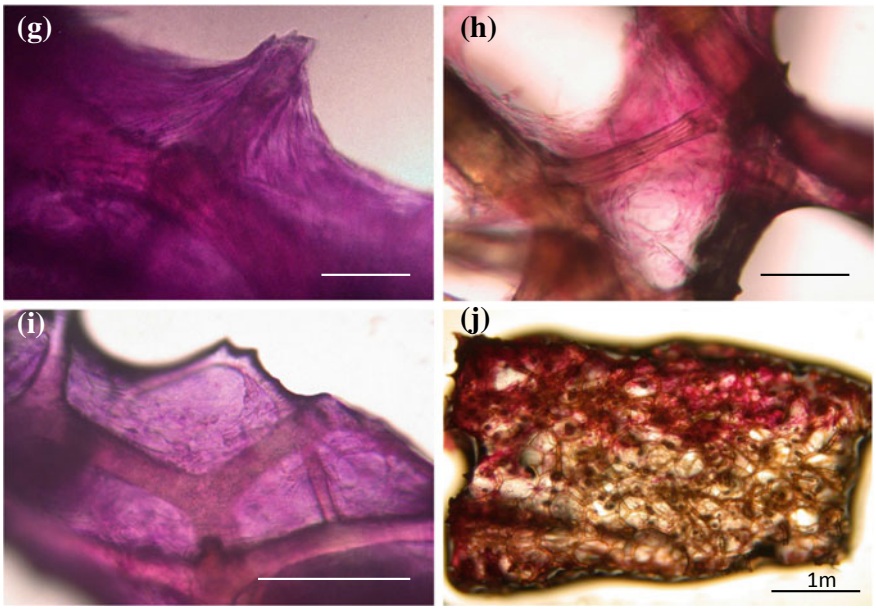

Fig. 12.11 (continued)

animal implantation. After 12 weeks subcutaneous implantation in rats, gross examination of the implantation sites (Fig. 12.12) showed negligible inflammatory reaction to the unseeded sponge skeletons. However, some vascularization and fibrous encapsulation are seen at the site of the implants of Species 4 and 5 (Fig. 12.12d, e) indicating a response to foreign material. The skeletons of all species have not been fully absorbed into the surrounding tissue (Fig. 12.12). However, Species 2 showed approximately 40% reduction in size. The gross appearance of the tissue surrounding the implants closely resembles the tissue of the negative control, which had not been implanted with sponge (Fig. 12.12f). Von Kossa histological staining was conducted on the samples to assess the presence of calcium deposits, which are stained black. No black areas were observed in sponge samples excised from the rats; therefore, the Von Kossa staining was negative for skeletons of all sponge Species (Fig. 12.13a–e). Correct method of Von Kossa staining was confirmed with the positive staining of the control slide (rat embryo) which is demonstrated by the black areas of staining (vertebrate) (Fig. 12.13f). There were strong similarities in the organization of cellular infiltration between the skeletons of each sponge Species (Fig. 12.13a–e). Pathological changes within the implantation sites, such as fibrosis, necrosis and the presence of foreign debris, were also observed to be similar between Species (Table 12.1).

Figure 12.14 shows the average inflammatory cell counts of each species of sponge skeleton and a negative control (no sponge implantation). As was stated in the methods, a score of 0.5 indicates the presence of 1–9 inflammatory cells, a score of 1

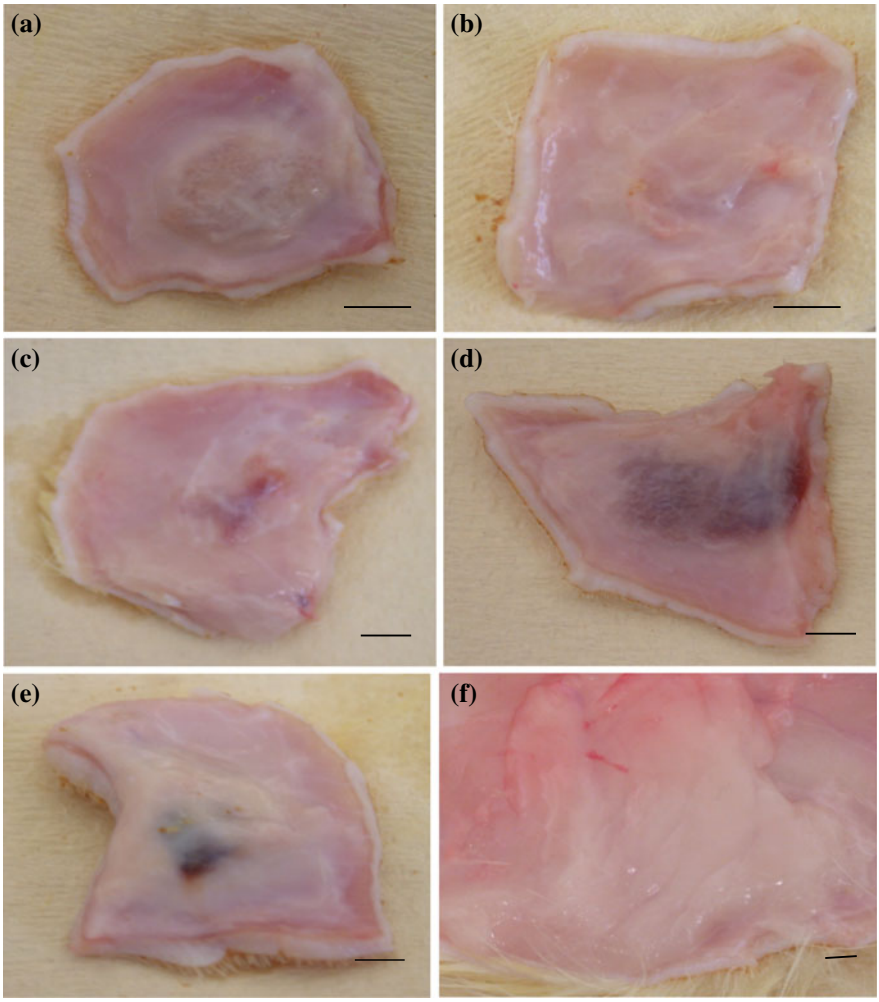

Fig. 12.12 Gross appearance of the scaffold site and surrounding area of implantation after 12 weeks *in vivo*. **a** ***Species 1*** showing the visible scaffold on the tissue surface; **b** ***Species 2***; **c** ***Species 3***; **d** ***Species 4*** showing vascularization of the scaffold and surrounding tissue; **e** ***Species 5*** showing vascularization of tissue and fibrous encapsulation of the implantation site; **f** Negative control, tissue which was not implanted with a scaffold sample (Scale bar = 5 mm)

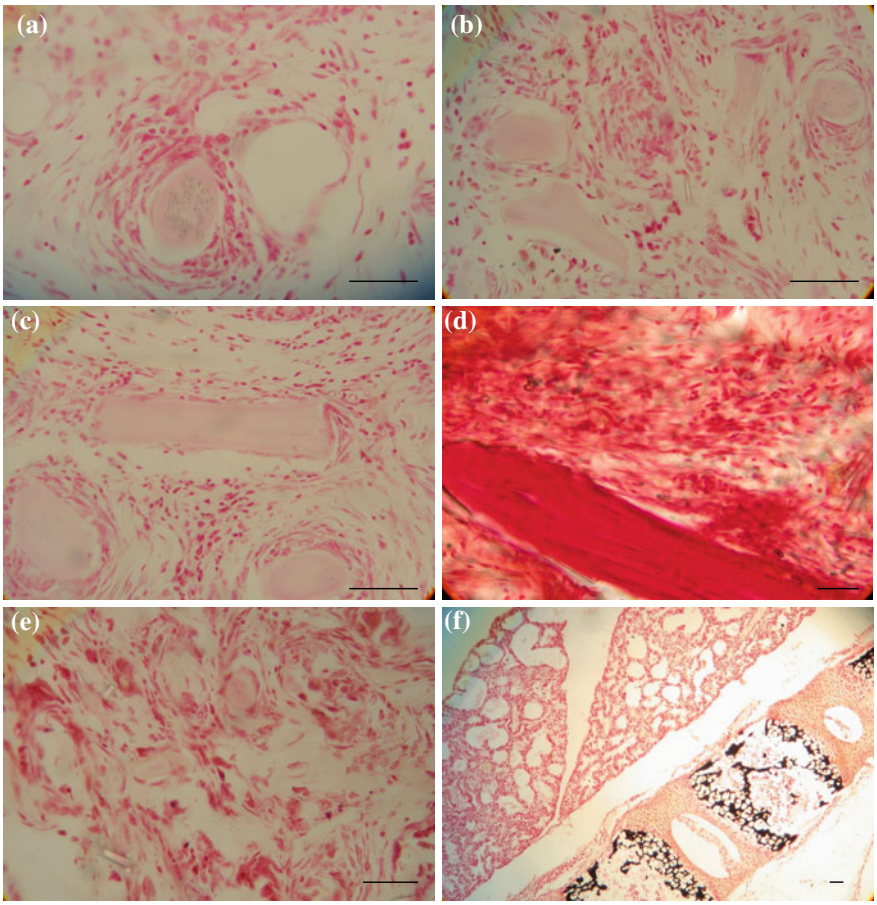

Fig. 12.13 Von Kossa staining of sponge scaffolds after 12 weeks implantation, and a control slide. **a** ***Species 1***; **b** ***Species 2***; **c** ***Species 3***; **d** ***Species 4***; **e** ***Species 5***; **f** Control (rat embryo) (Scale bar = 40 μm)

indicates the presence of 10–50 cells. Infiltrated inflammatory cells showed a predominance of mononuclear cells. The average inflammatory cell counts for Species 4 and 5 are higher than those for Species 1, 2 and 3 (Fig. 12.14), however statistical analysis (Student-Newman-Keuls) showed no significant difference between the inflammatory responses of all sponge skeletons.

Table 12.1 Pathological response

Samples	Fibrosis		Haemorrhage (3)	Necrosis (4)	Degeneration (5)	Foreign debris (6)
	Fibrosis (2a)	Fatty infiltrate (2b)				
Species 1						
Sample 1	+++	++	–	–	–	–
Sample 2	+++	+	+	–	–	–
Sample 3	+++	++	–	–	–	–
Sample 4	+++	–	–	–	–	–
Sample 5	+++	+	+	–	–	–
Species 2						
Sample 1	+++	+++	–	–	–	–
Sample 2	+++	++	–	–	–	–
Sample 3	+++	+++	–	–	–	–
Sample 4	+++	+++	–	–	–	–
Sample 5	+++	+++	–	–	–	–
Species 3						
Sample 1	+++	++	–	–	–	–
Sample 2	++	–	–	–	–	–
Sample 3	+++	+++	–	–	–	–
Sample 4	+++	++	–	–	–	–
Sample 5	++	+	–	–	–	–
Species 4						
Sample 1	++	+	–	–	–	–
Sample 2	+	+	++	–	–	–
Sample 3	+	–	+	–	–	–
Sample 4	++	++	–	–	–	–
Sample 5	+	+	+	–	–	–
Species 5						
Sample 1	++	–	+	–	–	–
Sample 2	+++	++	–	–	–	–
Sample 3	+++	++	++	–	–	–
Sample 4	+	+	++	–	–	–
Sample 5	++	++	–	–	–	–

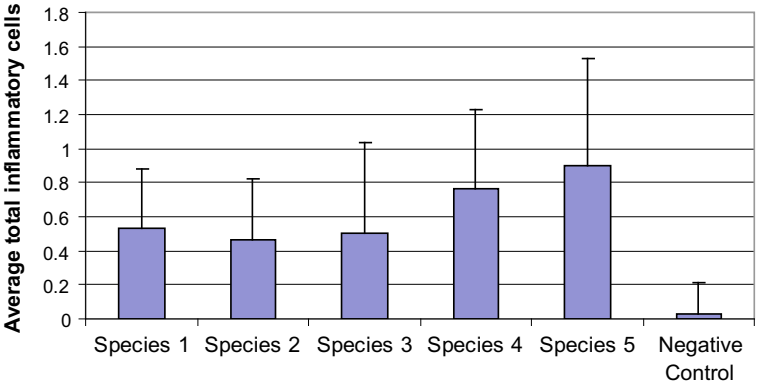

Fig. 12.14 Graph showing the inflammatory response (average total inflammatory cells whereby 0.5 indicates 1–9 cells, and a score of 1 indicates 10–50 cells) of each sponge Species on rat surrounding tissue after 12-week subcutaneous implantation in rats

12.7 Conclusion

Identification of suitable scaffolds onto which stem cell and progenitor cells can be seeded to generate functional three-dimensional tissues is a major research goal. Marine sponge skeletons were assessed for use as bioscaffolds on the basis of their collagen fiber extracellular matrix, interconnecting canal systems forming porosity, ability to hydrate to a high degree, and the diverse skeletal architectures within the phylum *Porifera*. This study indicates that the collagenous fiber skeleton of marine sponges provides a suitable bioscaffold for cell-based bone tissue regeneration as it supports the adhesion, migration and proliferation of osteoblasts in vitro. Importantly, the osteoblastic phenotype of cells was confirmed by staining for alkaline phosphatase, an early phenotypic marker of the osteoblast. Together with preliminary biocompatibility studies, this study indicates that natural marine sponge skeletons may offer a potential new source of bioscaffold for the repair of bone defects. This project supports the study by Green et al. [4], advocating sponge collagen as a bioscaffolding material for bone tissue engineering. The contrasting degrees of cellular attachment and proliferation on these five different species of *Porifera* skeletons illustrate the ability of pore dimension, microstructure, nanostructure, permeability, and composition of scaffolds to influence cellular processes. The abundance and structural diversity of natural marine sponge skeletons, and their potential as osteoinductive and osteoconductive frameworks, indicate a promising new source of scaffold for tissue regeneration. This study additionally indicates that marine sponge skeletons provide useful models for future experimentation in scaffold design (biomimicry), whilst suggesting the possible potential of these materials for osseous treatment.

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Chapter 13
Chitinous Scaffolds from Marine Sponges for Tissue Engineering

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Abstract Chitin as a biological material which has been identified in skeletal structures of a broad variety of unicellular (yeast, protists, diatoms) and multicellular (sponges, corals, worms, molluscs, arthropods) organisms is recognized as natural template with good perspectives in modern biomedicine. This chapter provides first insights into prospective applications of naturally prefabricated three-dimensional chitinous scaffolds from selected marine sponges in tissue engineering. This became possible only owing to the recent discovery of poriferan chitin which provoked

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renewed multidisciplinary interest driven by growing demand in novel biomimetic materials. Here, we focused on both demosponges of Verongiida order as a renewable source of chitin scaffolds with jewelry designs, and human mesenchymal stromal cells having high therapeutic potential. The chapter covers approaches for isolation of scaffolds from the chitin-bearing marine sponges, nuances of their interaction with human cells and cryopreservation potential.

Keywords Marine sponges · Chitin · Chitinous scaffolds · Tissue engineering · Stem cells · Cytocompatibility · Biomimetic approaches · Cryopreservation

13.1 Introduction

Tissue engineering is a multidisciplinary research field based on principles of engineering and biology with the final goal to design functional tissue bioequivalents for subsequent grafting and regeneration of damaged tissue [1]. Recent trends in this highly promising discipline are keeping pace with the growing clinical demand of new scaffolding materials worldwide (reviewed in [2]). Both synthetic and natural biomaterials offer a broad complementary spectrum of characteristics one may choose to design scaffolds with target application requirements (for review see [3]). However, to pre-shape the biomaterials into bulk three-dimensional (3D) structures and confer their appropriate structural and functional advantages, technically complex fabrication processes are necessary. In this regard, marine sponges represent a rich source of evolutionary refined 3D scaffolds with unique properties for biomedical application [4, 5] which makes them attractive candidates in particular for bone tissue engineering applications [6]. However, their overall tissue engineering potential is still largely underexplored.

Chronologically, first reports addressing cytocompatibility of skeletons isolated from marine sponges with mammalian cells were described for so-called commercial sponges, also known as bath sponges. According to Laubenfels and Storr, "the commercial sponge is the macerated and dried skeleton of one of the sponge animals that has no proper spicules. It must be from a species whose skeleton consists of spongin fibers, and furthermore, these fibers must continue to be elastic or 'spongy' even after having been dried" [7]. Intriguing historical information has been recently reported in the review by Jesionowski and co-workers: "successful attempts to use spongin in the form of commercial sponges as biomedical implants have been reported since the 18th century. Fragments of the bath sponge skeleton were used as small prostheses in early "plastic surgery". Revolutionary results can be found in the paper published by Hamilton in 1881 under the title "*On sponge-grafting*". In the reported case, a woman underwent surgery for the removal of a mammary tumor, during which a large area of skin was removed. This skin was replaced with a thin slice of aseptic sponge skeleton, which, ten days after the surgery, was observed to be vascular, and three months later, was covered with epithelial tissue" [8].

Table 13.1 Examples of cytocompatibility of collagenous marine sponges with mammalian cells

Sponge species	Type of mammalian cells	Reference
Species of *Spongia* (later classified as *Spongia officinalis*)	Human osteoprogenitor cells	[9]
Chondrosia reniformis	Mouse osteoblasts (immortalized cell line 7F2)	[12]
Unidentified sponge species of the genus Hippospongia, the genus Callyspongia, and the family Chalinidae	Mouse calvarial-derived primary osteoblasts	[13]
Spongia officinalis	Human bone marrow stromal cell; human fetal tissue-derived mesenchymal stem cells	[14]
Family Callyspongiidae	Mouse primary osteoblasts	[15]
Spongia agaricina	Primary human endothelial cells derived from umbilical vein, human dermal microvascular endothelial cells, osteoblast-like MG63 cell line	[16]
Biemna fortis	MC3T3 (osteoblast precursor cell line derived from mouse calvaria)	[17]
Spongia agaricina	Human foetal osteoblasts	[18]
Petrosia ficiformis, *Agelas oroides* and *Chondrosia reniformis*	Immortalized mouse lung fibroblast cell line (L929)	[19]
Chondrosia reniformis	L929 mouse fibroblast cell line, human keratinocyte cell line	[11]

In the recent literature, spongin-based sponges have been called "collagenous sponges" by David Green [9]. The direction to use such sponges for the aims of tissue engineering and biomedicine is well in trend up today (Table 13.1). In contrast to spongin-based sponges, such species as *Chondrosia reniformis* possess ability to synthetize collagenous networks (see for review [10]) with a good chance to be applicable in tissue engineering [11].

The later discovery of chitin in marine [20, 21] and freshwater [22] sponges opened not only a novel source of this attractive biomaterial but also a renewable source of unique 3D scaffolds [23, 24] due to the possibility to cultivate chitinous demosponges using aquaculture technologies. Richness and diversity of scaffold designs uncovered in chitin-producing sponges created numerous opportunities to advance their application in biomedical field. Analogous to silica-collagen biocomposites of poriferan origin [25, 26], their chitin counterparts [27] hold great promise due to increased mechanical stability and great variety of scaffold templates that could be easily adapted to tissue engineering needs using biomimetic approaches.

Demonstrated possibility to functionalize poriferan chitinous scaffolds with desired compound of biomedical interest as in the case with SiO_2-chitin [28, 29], ZrO_2-chitin [30, 31] and Fe_2O_3-chitin composites [32, 33] considerably increases

their tissue engineering value. The further development and standardization of purification protocols based on alkali-acid treatment led to discovery of chitin scaffolds in new sponge species, e.g., *Aplysina fistularis* [34]. Collectively, all this paved the way to first in vitro and in vivo cytocompatibility studies involving chitinous sponges and various cell types. For instance, it was shown that the scaffolds derived from *A. cauliformis* supported long-term growth and functionality of primary porcine and human chondrocytes. They were capable of depositing proteoglycan-rich collagen type II positive extracellular matrix in vitro as well as forming ectopic cartilage after subcutaneous transplantation into mice with severe combined immune deficiency [24]. This stimulated further investigations which demonstrated the cytocompatibility of Verongiida sponges *Aplysina fulva*, *Aplysina aerophoba* and *Ianthella basta* with human adipose tissue-derived MSCs [35]. The most recent identification of chitin in new Verongiida species such as *Pseudoceratina purpurea*, in non-Verongiid marine demosponges *Mycale euplectellioides* [36, 37] as well as in Red Sea demosponges sponges *Acarnus wolffgangi* and *Echinoclathria Gibbosa* [38] which can be cultivated at large scales using marine aquaculture stimulates future evaluation of their tissue-engineering capabilities.

13.1.1 Chitinous Scaffolds from Marine Sponge Ianthella Basta: Prospects for Tissue Engineering

Decellularizaion of three-dimensional organ matrix and its repopulation using stem cells is a fundamental concept of tissue engineering [39]. Acellular matrix can act as a natural platform providing mechanical support and microenvironment required for cell infiltration, proliferation as well as differentiation into target tissues. From a number of marine sponges in which chitin has been discovered, one species of so called “elephant ear sponge” *Ianthella basta* deserves particular attention for its chitinous matrix structurally defined in a unique manner [40]. This matrix can be easily isolated and characterized in a controllable manner using standardized processing workflow as proposed by Ehrlich et al. [20]. This method ensures complete removal of sponge cellular debris and inorganic components while maintaining structural integrity and biomechanical properties of scaffold framework. One commercial prerequisite for *Ianthella basta* application in tissue engineering is its big size and relatively high growth rates. *Ianthella basta* colonies reaches sizes up to 35,000 cm^2 in its natural environment in the Indo-Pacific region [41].

Figure 13.1 shows representative pictures of original *Ianthella basta* sample (a) and purified chitinous framework made up of numerous repeating units of square-shape macropores with the diameter up to 2 mm (b). Such gauze bandage-like scaffold geometry and a broad use of chitin in production of wound healing coatings [42] make these scaffolds attractive especially for wound management. Excellent hemo- and bacteriostatic activity of chitin successfully demonstrated in numerous animal and

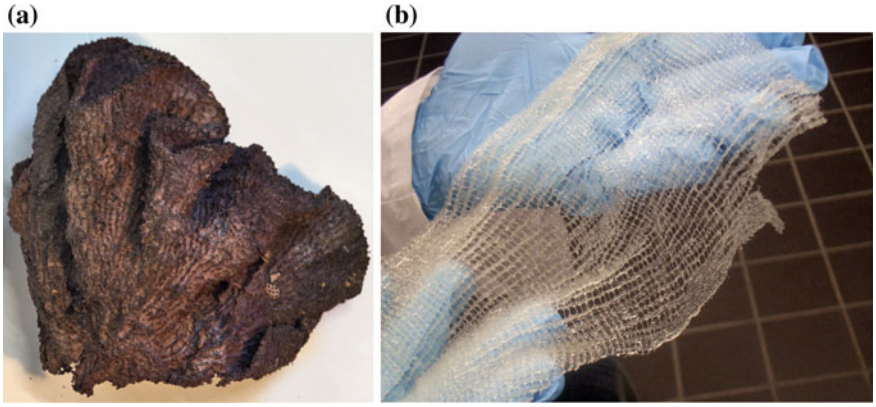

Fig. 13.1 Photograph of 15 cm-large fragment of freeze-dried *Ianthella basta* demosponge as collected (**a**) and bulk chitinous 3D scaffold obtained after step-wise purification procedure (**b**) which resembles the original shape of the sponge skeleton. The building block of the mesh is represented by square chambers firmly linked to each other via tubular, intercalated chitin microfibers (for details see Brunner et al. [40] and Ehrlich [4, 5])

clinical studies on wound healing (review in [43]), led to commercializing of chitin dressings such as Chitopack S®, Chitopack P®, Chitopack C® and Bechitin®).

Figure 13.2 is an illustration of a single square shaped cell having complex capillary network formed by translucent interconnected chitin fibers (a) with open axial channels inside (b). Such a fiber composition and overall large internal surface area of sponge's skeletons enables considerable liquid absorption and transport of gases and nutrients throughout network by capillary forces (for details see [30]). This provides favorable microenvironment necessary for cell attachment and expansion as well as dynamic cell-cell and cell-matrix interactions within 3D chitinous matrix. Capillary-like structure of *Ianthella basta* fibrous scaffolds and overall beneficial effects of chitin on angiogenesis may trigger proper oxygenation and nutrient supply favorable for neovascularization which plays a crucial role in tissue engineering (see for overview [44]) and is very challenging [45]. We could further assume that remarkable fluid-absorbing properties shown for marine sponges biopolymers-based skeletons may be most likely applicable to wound healing field due to effective absorption and retention of wound exudates.

Sterilization is an essential step in manufacturing of tissue-engineered scaffolds to provide contamination-free in vitro cultivation in cell culture plates or bioreactors and engineered products of high quality [46], ideally, not compromising scaffold composition and mechanical properties. Chitin from marine organisms is a highly thermostable material having the range of the thermal degradation of 300–460 °C [47] and, because of that, it is extensively used as a versatile template for extreme biomimetics approaches (for review see [48]). Furthermore, according to the literature chitin has high glass transition temperature around 335 °C [49] and is insoluble in most regular organic solvents. In our disinfection studies, we used autoclaving

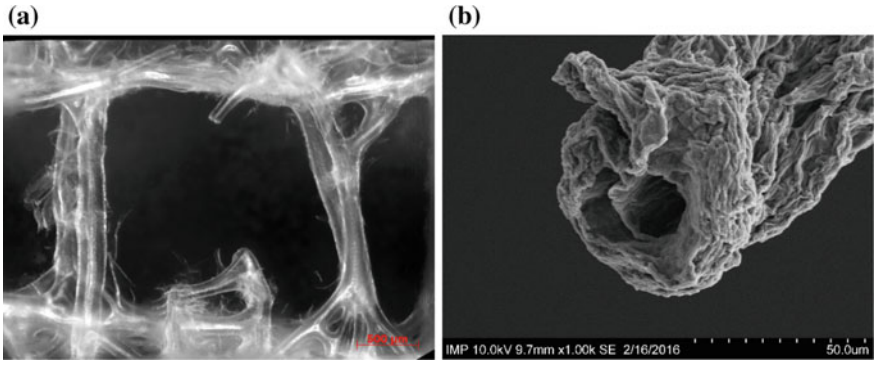

Fig. 13.2 Microimagery of *Ianthella basta* chitinous scaffold. Stereomicroscope image of a typical scaffold cell composed of translucent capillary-mimicking chitin skeletal fibers (**a**) and SEM image of a single chitin fiber with a well discernable internal axial channel. Such composition of poriferan chitin structural elements may favor nutrient supply for cell ingrowth in 3D environment

[50], 70% ethanol and supercritical carbon dioxide treatments [51]. Neither damage to chitinous scaffolds derived from *Ianthella basta* nor change of their cytocompatible properties was observed after sterilization. Moreover, they were stable in long-term culture.

As the next step, such sterilized scaffolds were seeded with human mesenchymal stromal cells (hMSCs) and their viability was determined using live-dead staining. As can be seen from Fig. 13.3, chitinous scaffolds from *Ianthella basta* are non-toxic to cells on day one (a) as well as day 14 (b) after seeding. An increase in the number of marked cell colonization on the scaffolds as time progresses suggests there are biological cross talks, which supports high cell viability and promoting cell proliferation. Such 3D platforms constructed from chitinous scaffolds and selected human cells may serve as an ex vivo engineered skin model for drug screening and cosmetic testing that would better mimic physiological conditions compared to cell expansion in 2D monolayer culture [52]. To support this hypothesis, recent publication revealed that alpha-chitin nanofibers and nanocrystals prepared of crab shell have protective effect on epithelial cells and reduce proinflammatory cytokine content as demonstrated in 3D human tissue-engineered skin model [53]. Later, the same research group reported that chitin nanofibers have not only anti-inflammatory and antioxidant properties but also are protective against ultraviolet radiation [54]. The similar future research on anti-inflammatory effects of poriferan chitin is of high fundamental and practical importance.

Composition, stiffness, nanotopography and specific biomechanical signals provided by certain 3D scaffolds types govern alignment, proliferation, migration and, importantly, differentiation of stem cells to certain lineages (for review see [55]). All these characteristics are important to consider in order to design scaffolds with suitable microenvironment in which cells can reside and acquire corresponding phenotypic patterns. In our experiments with chitinous scaffolds from *Ianthella basta*,

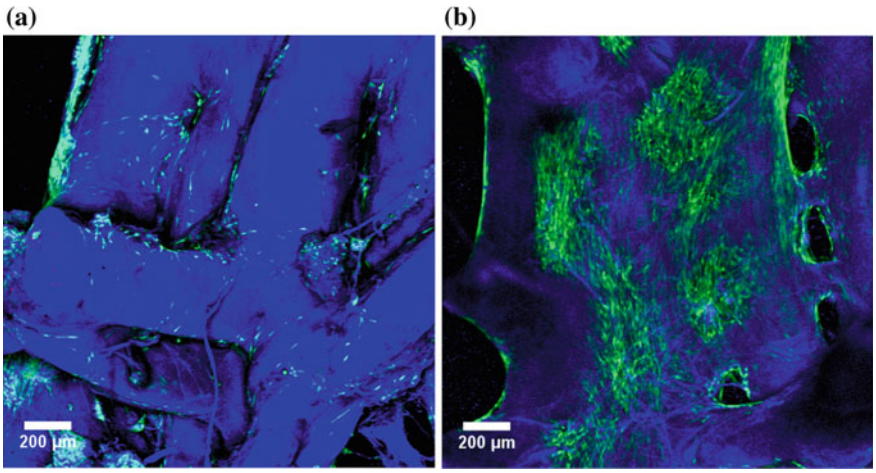

Fig. 13.3 Representative fluorescent images of chitinous scaffolds derived from *Ianthella basta* seeded with hMSCs on day one (**a**) and day 14 (**b**) in culture. Chitin is stained with specific dye Calcofluor white and exhibits intensive blue fluorescence even after very short light exposure time (lower than 1/500 s). Live cells are stained with fluorescein diacetate and exhibit green fluorescence. Dead cells are stained with ethidium bromide and have red fluorescence. Only few dead cells, spread green cells and visible increase in cell number with time indicates excellent scaffold cytocompatibility with hMSCs

two differentiation lineages adipogenic and osteogenic, were successfully generated. Figure 13.4a demonstrates sheets of adipogenic-stimulated hMSCs in 3D chitinous scaffolds with clearly discernable lipid droplets characteristic for successfully differentiated adipocytes. Similarly, Fig. 13.4b displays sheets of osteogenic-differentiated cells expressing alkaline phosphatase differentiation marker. Such formation of tissue-like structures with differentiated cells could be used for studying differentiation in 3D culture systems compared to cell monolayer cultures.

New advances in transplantation of stem cells, tissues and organs require development of efficient methods for their long-term storage [56]. Cryopreservation process usually includes incubation of a sample with cryoprotective solution, cooling to subzero temperatures, storage for a certain period of time, thawing, and removal of the cryoprotectants and recovery of cells under physiological conditions.

During cooling, crystallization event occurs which in turn comprises of ice nucleation and crystal growth. In this context, we were the first who reported on cryopreservation of human cells within scaffolds derived from marine sponges [57] with a moderate cell recovery following cryopreservation (higher than 50%). These results were significantly improved in follow-up studies using sucrose as extracellular cryoprotectant in combination with dimethyl sulfoxide (DMSO) as an intracellular one (unpublished data). Similar results were reported on cryopreservation efficiency of engineered epithelial sheets based on keratinocytes seeded and frozen on chitosan-gelatin membranes [58] where DMSO-based cryopreservation was con-

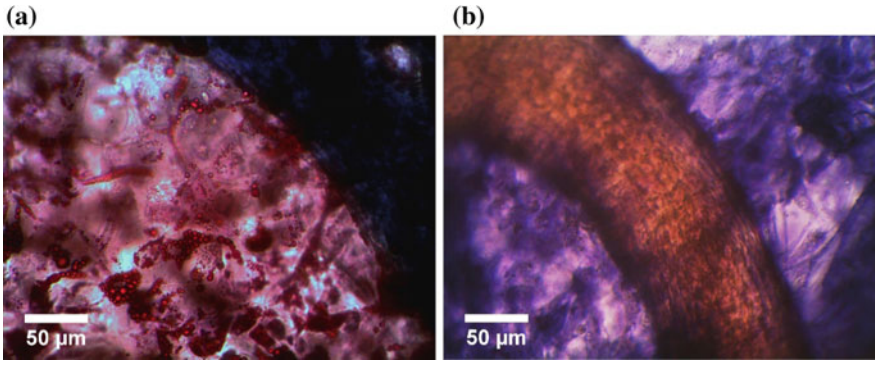

Fig. 13.4 Light microscopy pictures of hMSCs, those differentiation towards adipogenic and osteogenic lineages was induced. Accumulation of lipid droplets is visualized by bright red color after classical Oil Red O staining (**a**) and alkaline phosphatase gives intense blue color after Fast blue staining (**b**) indicating early osteogenesis, respectively. This finding shows that easy-to-isolate chitinous scaffolds from *Ianthella basta* support assembly of hMSCs into cell sheets in long-term culture and promote their multilineage differentiation in this unique naturally prefabricated 3D microenvironment

siderably improved through the introduction of another disaccharide trehalose. In our cryomicroscopy studies, we did not reveal any dramatic changes in scaffold structure after cryopreservation utilizing 10% DMSO (Fig. 13.5).

The main rational behind cryopreservation studies in tissue engineering implies the ability to prepare functional tissue substitutes in advance and develop effective storage strategies for their retrieval upon demand in addition to enhancing the logistics by implementing continuous 'in-line process'. Interestingly, it has recently been shown that inherent properties of a material, more specifically its glass transition temperature and alignment of scaffold fibers as well as viscoelastic properties, drastically influence the fate of cryopreserved adherent cells [59]. The authors showed the interesting observation that increase in scaffold elasticity mitigates differential thermal contraction as compared to more stiff scaffolds of the same material and thereby enhances post-thaw cell recovery. Consequently, to prevent excessive shrinkage during freeze-thawing, the elasticity of poriferan scaffolds could be adjusted by modifying purification parameters and it is a point worth investigating. Other promising cryopreservation tools such as vitrification and directional freezing can be examined on *Ianthella basta* scaffolds for the preservation of stem cells in 3D environment [60].

Thus, we showed that demosponge *Ianthella basta* due to its ability to produce unique, flat 3D scaffolds (Fig. 13.1b) is an appealing objective for tissue engineering applications. The feasibility of culturing these sponges in sea-ranching conditions and their high growth rates may provide a sustainable source of chitinous porous scaffolds. These scaffolds could be purified and produced with different size and shape in a controlled manner, sterilized and seeded with corresponding stem cells. The derived chitinous scaffolds support attachment, proliferation and differentiation

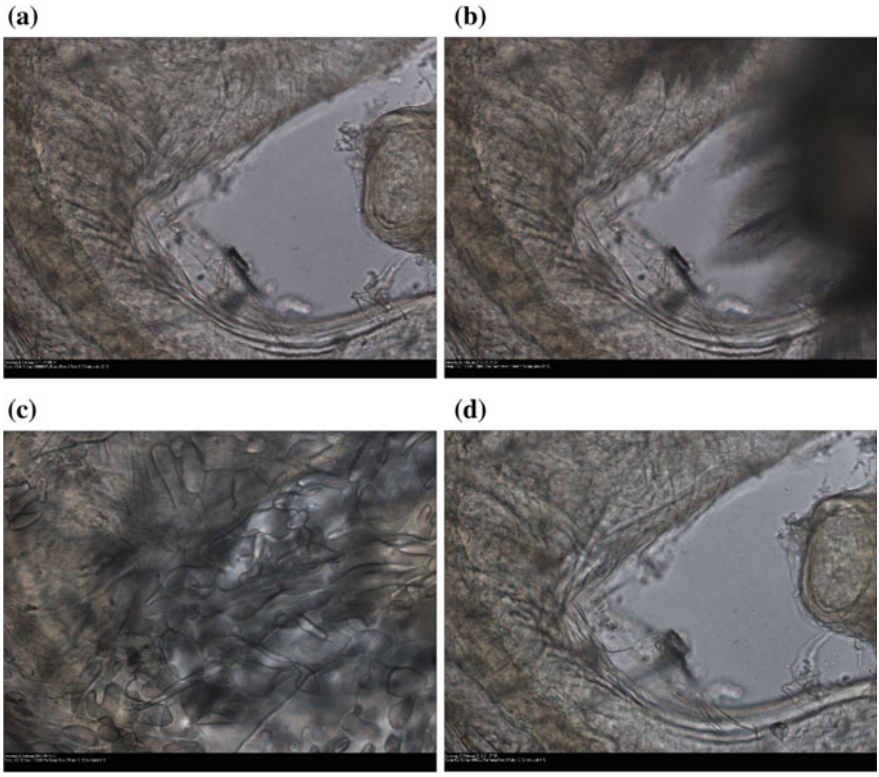

Fig. 13.5 Cryomicroscopy images of the cell-free chitinous scaffolds derived from *Ianthella basta*: **a** before the ice nucleation; **b** nucleation event at −13 °C with typical dendritic ice crystals; **c** ice melting at −3 °C; and **d** after thawing back to +4 °C. No apparent alterations in scaffold shape and structure after thawing have been observed

of hMSCs and should be attractive as wound dressings, 3D drug screening platforms as well as cryobiological models.

13.1.2 3D Chitinous Scaffolds from Marine Sponges Aplysina Aerophoba and Aplysina Fulva: Interspecies Biocompatibility with Human Cells

Another area of interest to many multidisciplinary research teams is motivated by the fascinating microarchitecture and structural properties of other demosponge *Aplysina aerophoba* belonging to *Aplysinidae family* (order Verongiida). These sponges represent a renewable source of scaffolds due to their abundance and standardized cultivation in laboratory and mariculture, for instance, in the Adriatic Sea [61]. In particular, in Boka Kotorska Bay of the Adriatic Sea, significant amounts

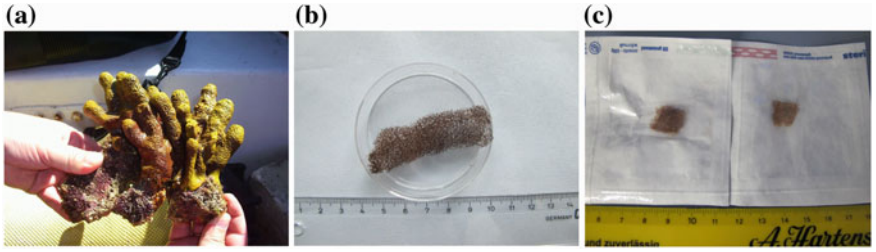

Fig. 13.6 Technological route for derivation of chitinous scaffolds from *Aplysina aerophoba* and their preparation for subsequent in vitro culture. **a** *Aplysina aerophoba* fragment from the marine farming facility after collection in the Adriatic Sea (Kotor Bay, Montenegro); **b** Purified 3D skeleton of *Aplysina aerophoba* with preserved original shape; **c** Derived chitinous scaffolds in vacuum sealed packages before (left) and after (right) sterilization using supercritical carbon dioxide treatment

of this sponge could be obtained in a sustainable way. Furthermore, ready-to-use chitinous scaffolds from this sponge have recently become commercially available from BromMarin GmbH in Germany.

In a natural habitat, this sponge possesses finger-like cylindric body (Fig. 13.6a). After lyophilization of sponge skeleton, additional processing steps include stepwise removal of water-soluble salts and impurities, proteins and residual pigments, calcium and magnesium carbonates in respective treatment solutions [23]. Such isolation results in obtaining bulk volumetric mesh (Fig. 13.6b) which could be subsequently sterilized, for example, by subjecting to supercritical carbon dioxide treatment (Fig. 13.6c). This technique is highly effective for sterilization of decellularized biomaterials and does not exert any damaging or cross-linking impact on them [62].

To better comprehend the structural features of the scaffolds obtained from *Aplysina aerophoba*, a more detailed characterization of fiber morphology is provided hereinafter. SEM analysis revealed that the resulting macroporous finger-like latticework of *Aplysina aerophoba* has a very complex naturally branched spatial design (Fig. 13.7a) and is totally different from a more planar *Ianthella basta* scaffolds. Morphologically, the derived scaffolds resemble human cancellous bone. The stratified composition of chitinous fiber clearly visible on Fig. 13.7b gives reasons to assume that its external layers might provide substrate for cell anchoring and distribution while the inner layers endows fibers with increased mechanical strength. The biomimetic strategies aiming at producing multi-layer scaffolds are increasingly being used in tissue engineering, for example, for musculoskeletal regeneration or small intestine [63] to mimic native layered tissue [64]. The presence of microchannels within the fibers of *Aplysina aerophoba* chitin (Fig. 13.7c) and apparent fiber interconnectivity may ensure effective circulation of nutrients over the chitinous ducts sufficiently deep into central parts of the scaffolds to facilitate corresponding cell ingrowth. The complete diversity of intriguing structural peculiarities of scaffolds isolated from *Aplysina aerophoba* demosponge convinced us to evaluate their behavior in vitro.

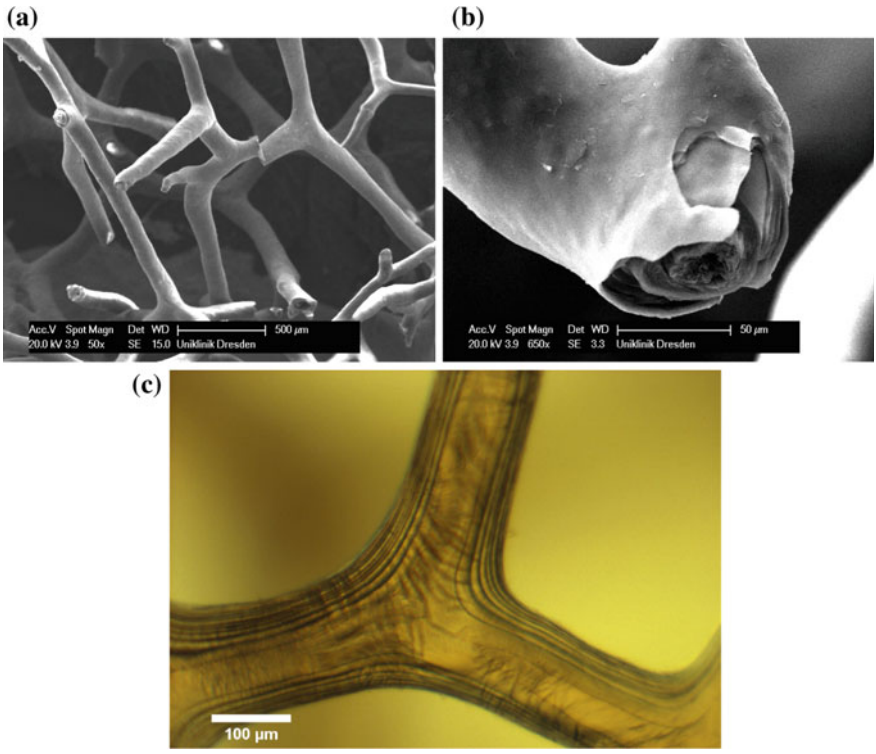

Fig. 13.7 Detailed representation of *Aplysina aerophoba* chitin scaffold microstructure. **a** SEM image of spatial arrangements of interconnected chitinous fibers as the basic structural matrix of the 3D scaffold; **b** SEM image of an individual chitinous fiber with typical radially oriented multilayers. Bright field image (**c**) shows the internal structure of selected chitinous fiber with tubular inner part

Cell adhesion is a fundamental feature which serves for cell communication with a substrate and other surrounding cells and is a pivotal requirement to be met when designing 3D scaffolds (for review see [65]). Moreover, exploring how cells adhere, flatten and spread in a 3D environment are more appropriate compared to 2D culture in an attempt to understand cell behavior in physiological conditions. It is known that in a standard monolayer culture, cells possess a lower vertical height whereas in a 3D scaffold cells are able to spread in all three dimensions (for review see [66]). The overall affinity of a cell for a scaffold determines its future fate achieved through cell migration, proliferation and differentiation. As synthetic materials may lack sites for cellular adhesion, they are often functionalized using specific attachment-enhancing molecules, treated by a variety of chemicals to modify their surface or to increase scaffold hydrophilicity [67]. In the context of decellularization, an adequate control over the treatment type and time is critically important in order to preserve cell adhesion sites for target cell populations, their differentiation capability and recapitulate a functional target organ. Common methods for enhancement of cell

attachment to decellularized tissues are conjugation of bioactive molecules such as antibodies and RGD peptides [68]. Irrespective of considerable structural difference between *Ianthella basta* and *Aplysina aerophoba*, similar positive tendency in cell-scaffold interactions was detected from the first in vitro studies. This eliminates any need in cost-consuming modifications of the scaffold surface to increase efficiency of attachment at least for hMSCs. Specifically, confocal microscopy shows high cellular viability in the core and peripheral regions of chitinous scaffolds on day 7, which is a clear evidence of the scaffold non-toxicity (Fig. 13.8a). In addition, Fig. 13.8b presents intact cellular cytoskeleton and numerous cell nuclei reflecting proliferation and propagation of hMSCs throughout intricate labyrinth of naturally prefabricated chitinous fibers. Moreover, as shown on Fig. 13.8c cells were able to attach, spread and migrate when using fibrous network as a solid support for further colonization of this scaffold. It is relevant to note that in these experiments hMSCs have been seeded using perfusion method to provide better cell saturation of the decellularized chitinous scaffolds.

Surprisingly, the quality and rate of hMSCs growth over the decellularized chitinous skeletons of *Aplysina aerophoba* was totally different from that of *Ianthella basta*. As depicted in Fig. 13.9, at around day 14 typical cell assemblies stretching between fibers appears (a) and by day 21–28 (depending on the initial seeding density), they almost completely refill the available voids between the chitinous fibers (b). We strongly believe that when using dynamic culture in perfusion bioreactor systems, 3D expansion of such pre-seeded constructs would even more accelerate the cell growth efficiency analogous to what was shown for synthetic scaffolds [69].

This assumption is expected because marine sponges live under constant water flow and their exoskeletons, in terms of tensile strength, are adapted to withstand strong waves and currents as well as any resultant shear stresses present. Cell bridging phenomenon might be related to variable 3D organization and biomechanics which distinguish these Verongiid demosponges morphologically from one another. This type of cell connectivity and migration is not fully understood and definitely merits in further investigation. In case of synthetic scaffolds, they would require laborious modifications to achieve such cellular communication and formation of cell-scaffold channels [70]. Thus, chitinous scaffolds from *Aplysina aerophoba* raise many fundamental biological questions and could find application as alternative 3D tissue-engineered model for instance to reduce animal usage in research and drug testing (for review see [71]).

Previously, we have revealed the multilineage differentiation capacity of hMSCs sheets grown within chitinous scaffolds derived from *Aplysina aerophoba* cultivated via marine ranching [72]. Such cells were able to differentiate into three canonical lineages, i.e. adipogenic, osteogenic and chondrogenic. Representative pictures of hMSCs with adipogenic phenotype characterized by lipid droplet accumulation in cell cytoplasm (Fig. 13.10a) and osteogenic phenotype (Fig. 13.10b) characterized by calcium deposition are shown below.

Maintenance of the differentiation potential of hMSCs seems to be a distinctive property not only for *Ianthella basta* and *Aplysina aerophoba* but also for many other Verongiida sponges. Here, for comparison purposes, we would like to high-

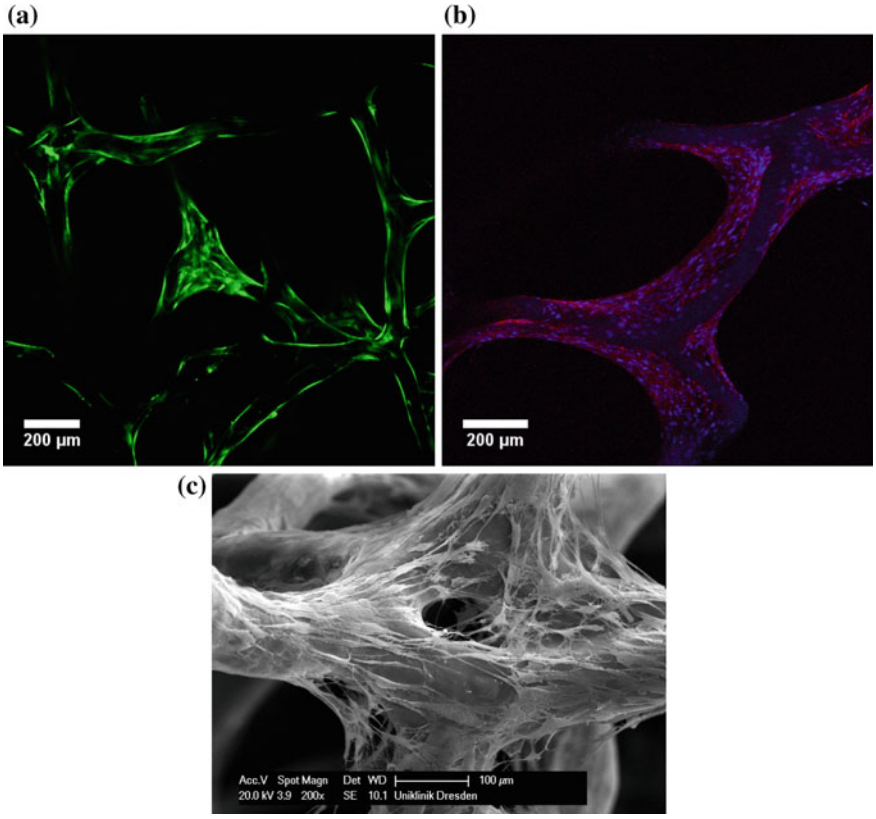

Fig. 13.8 Representative confocal fluorescence microscopy images (**a**, **b**) and SEM image (**c**) of the cell-seeded *Aplysina aerophoba* chitin scaffolds on day 7 in culture as a proof of interspecies compatibility of poriferan chitin with selected human cells. **a** Live-dead cytotoxicity assay shows high survival rate of hMSCs when grown on the chitinous scaffolds. Viable and dead cells are depicted in green and red, respectively; **b** DAPI/Phalloidin staining of cell nuclei and cytoskeleton. F-actin is shown in red and cell nuclei are counterstained in blue; **c** SEM analysis of cell attachment and spreading suggests that neither purification procedure nor poriferan chitin per se elicit any negative impact on human cells used in the study

light the exciting structure [23], phylogeny [73], and excellent biocompatibility of another demosponge *Aplysina fulva* (Verongiida: Demospongiae). The reticulate structure of this sponge mimics trabecular bone with a naturally diversified porosity (Fig. 13.11a, b). Furthermore, hMSCs were able to attach (Fig. 13.11c) and proliferate filling interfibrillar gaps between chitinous fibers from peripheral to central scaffold compartments. They were homogeneously distributed and characterized by enhanced alkaline phosphatase activity upon osteogenic stimulation (Fig. 13.11d).

From design and biocompatibility point of views, chitin scaffolds from both *Aplysina aerophoba* and *Aplysina fulva* demosponges are potential candidates for bone tissue engineering applications. In this regard, the organic part of their skele-

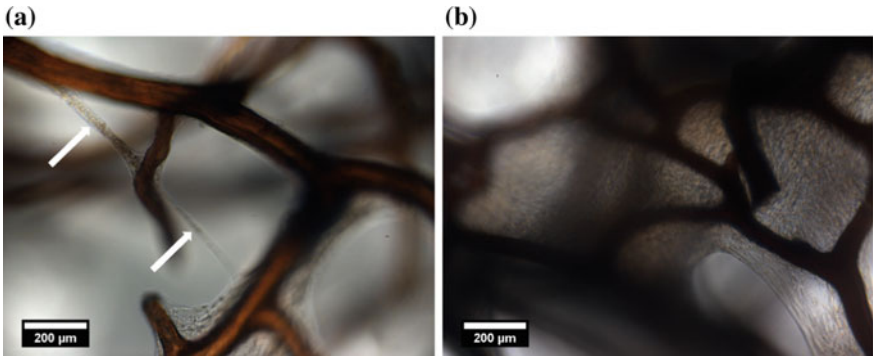

Fig. 13.9 Light microscopy images showing population of scaffolds derived from the skeletons of *Aplysina aerophoba* with selected hMSCs. White arrows indicate formation of bridging cell sheets between chitinous struts (**a**) which further develop into continuous membranous sheets wrapping the scaffold framework (**b**)

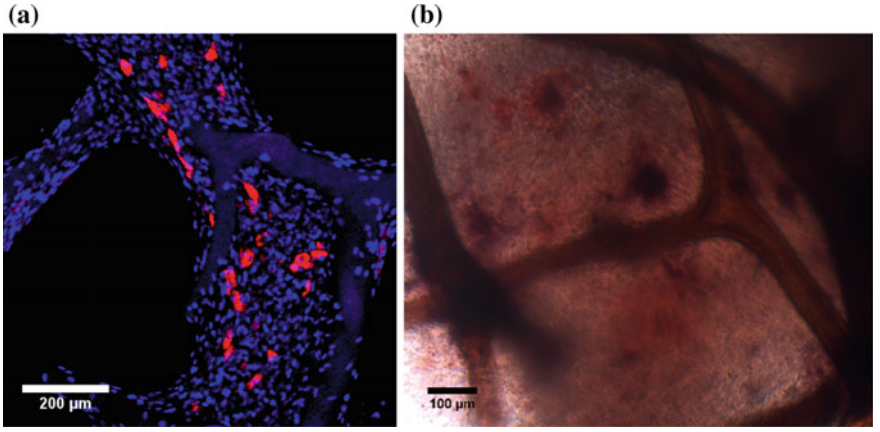

Fig. 13.10 Confocal fluorescence microscopy image (**a**) of adipogenic-induced hMSCs accumulating lipid droplets stained with Nile Red (red fluorescence); cell nuclei are counterstained with Hoechst (blue fluorescence). Bright-field microscopy image (**b**) of osteogenic-induced hMSCs sheets stained with Alizarin Red S contrasting calcium deposits by deep red color. Successful adipogenic and osteogenic differentiation further confirms the biocompatibility of this chitin scaffolds

tons can be functionalized with osteogenically active components of their inorganic part such as biosilica. For example, biogenic polymers polyphosphate and biosilica of poriferan origin were shown to be beneficial for application as biomimetic bone substitution materials (for review see [74]). These molecules exert osteoinductive and morphogenetic effects on cells revealed by significant increase in the expression of alkaline phosphatase and bone morphogenetic protein 2 [75]. Apart from that, it was demonstrated that polyphosphate induces accelerated tube formation of HUVEC (human umbilical vein endothelial cells) and, therefore, could be used in preparation

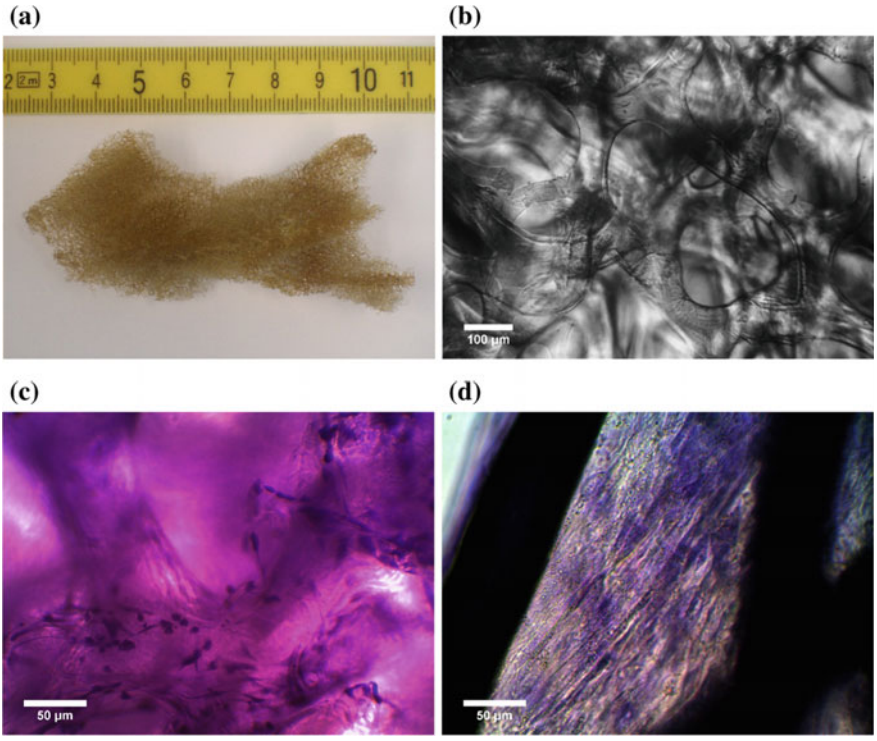

Fig. 13.11 General appearance of a cell-free skeleton from *Aplysina fulva* which was harvested in the Caribbean Sea and decellularized using alkali treatment (**a**); light microscopy image of uniform chitinous construct without cells (**b**), with hMSCs stained with Azur-Eosin (**c**) and for alkaline phosphatase on day 14 of in vitro culture (**d**). Chtinous scaffolds from *Aplysina fulva* roughly resemble trabeculae of spongy bone and support osteogenic capability of hMSCs

of bone tissue substitutes with intrinsic vascularization potential [76]. Gaharwar et al. [77] as well as Yang et al. [78] reported the positive effect of silica nanoparticles on induction of osteogenic differentiation of hMSCs. In these publications, the activity of alkaline phosphatase was significantly enhanced due to cellular uptake of silica nanoparticles as revealed in 2D cell culture studies. Likewise, similar results were observed in relations to the stimulatory effect of silica-based nanoparticles on murine osteoblasts in vitro and enhancement of bone mineral density in mice in vivo [79]. Since controlled generation of silica-chitin composites has already been successfully established for numerous chitinous sponges such as glass sponge *Farrea occa* [21] or Verongiida sponges *Aplysina cauliformis* [29], *Ianthella basta* [28] and *Aplysina aerophoba* (data not published), further evaluation of their osteoconductivity is of great interest. Other important point is that chitin scaffolds with incorporated silica nanoparticles may hypothetically have better cryopreservation properties providing multiple nucleation sites and improved thermal conductivity. Both topics are envisaged in future multidisciplinary research projects.

Other interesting aspect associated with the use of biomimetic approaches in tissue engineering is the fabrication of scaffolds for 3D direct or indirect co-culture systems that reflects more realistically the in vivo situation it is trying to address (for review see [80]). In practice, chitinous scaffolds derived from *Aplysina aerophoba* and *Aplysina fulva* could be used as a support for generation of 3D co-culture of osteogenically pre-stimulated hMSCs with endothelial cells to study neovascularization process in natural scaffolds and develop cryopreservation protocols that, to our best knowledge, have not yet been reported for mammalian co-culture systems.

Yet another prospective application where chitinous scaffolds from *Aplysina aerophoba* and *Aplysina fulva* can be useful is anti-tumor drug testing (for review see [81]). Such systems could integrate 3D scaffolds and anti-tumor compounds derived from the same marine sponges, for instance, Aeroplysinin-1 from *Aplysina aerophoba* (for review see [82]). Interestingly, the results of a recent publication revealed pronounced effect when they examined the anti-tumorigenic and anti-metastatic activities of aeroplysinin-1 and isofistularin-3 derived from *Aplysina aerophoba* against pheochromocytoma, with aeroplysinin-1 [83].

This subsection show that chitinous scaffolds derived from demosponges *Aplysina aerophoba* and *Aplysina fulva* possess a number of exclusive properties making them promising for use in tissue engineering. Among them, scaffold macro- and microdesigns, cytocompatibility with human mesenchymal stromal cells and support of their multilineage capacity. Using biomimetic approaches, these properties could be further enhanced according to biomedical needs.

13.2 Conclusion

The macro- and micro-designs of poriferan chitin scaffolds, their cytocompatibility with human mesenchymal stromal cells and support of their multilineage capacity make sponge chitin to be the intriguing alternative to scaffolds made of the structural proteins (collagen, keratin, and spongin) as well as artificial (polymers) materials. Fundamental questions about the pathways of demosponges chitin biosynthesis and its relationship with key biochemical reactions involved in the biosynthesis of diverse secondary metabolites (i.e. bromotyrosines) in the same species are still unanswered. There is a lack of knowledge concerning the mechanisms of chitin halogenization and biomineralization under natural conditions. What are the functional roles of halogens and mineral phases for rigidification of chitin-based skeletons of verongiid demosponges? Also, the challenging task of isolating and application of such mineral-containing and mechanically more stable 3D chitinous scaffolds from selected sponges remains to be addressed. The key ways for oxygen-free methods for carbonization of 3D chitin constructs isolated from diverse demosponges ought to be investigated.

There is also a lack in comparative study on pores diversity within diverse species of Verongiida sponges with respect to their potential as highly appropriative chitinous scaffolds. Consequently, the ultimate goal of our research is to answer questions

about the existence of the relationship between specificity of pore size, stiffness and fiber density of poriferan chitin scaffolds with respect to the aims of modern tissue engineering.

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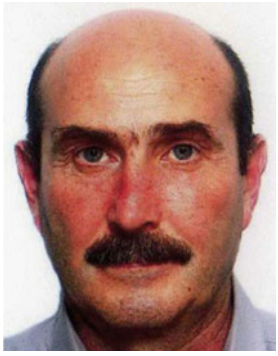

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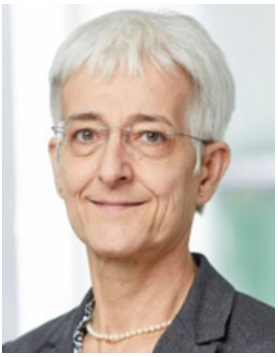

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Chapter 14
The Other Connective Tissue: Echinoderm Ligaments and Membranes as Decellularized Bioscaffold for Tissue Engineering

Kheng Lim Goh and Yos Morsi

Abstract This chapter examines the sea urchin ligament as a potential decellularized bioscaffold by discussing the significance of collagen fibrils, which are highly-paralleled slender structures embedded in the hydrated proteoglycan-rich (PG) extracellular matrix (ECM) of tendons, for reinforcing the soft connective tissue. The discussion is presented in two parts as follows. Part one examines the role of collagen fibrils for providing structural support for the tissue, in the context of the structure of the collagen fibril, and mechanics of stress transfer in the tissue. Part two will review the potential clinical applications of the decellularized bioscaffold, related to tissue implants for repair and regeneration.

Keywords Collagen fibrils · Taper · Extra-cellular matrix · Fibre composites

14.1 Introduction

Soft connective tissues, such as tendon, ligament and muscle, are biological examples of fibre reinforced composites. They share similar biomechanical functions. For instance, tendon, which connects bone to muscle, transmit the force from muscle to the bone during locomotion; ligament, which connects bone to bone, transmits force from one bone to the other [1]. The extracellular matrix (ECM) of these tissues also share similar structural features, comprising a blend of hydrated macromolecular assemblies of proteins and polysaccharides, collageneous fibrils, elastin fibres [2].

Collagen fibrils are the key components underpinning the reinforcement to the tissue, which is analogous to fibre reinforcement of a composite material [3–5].

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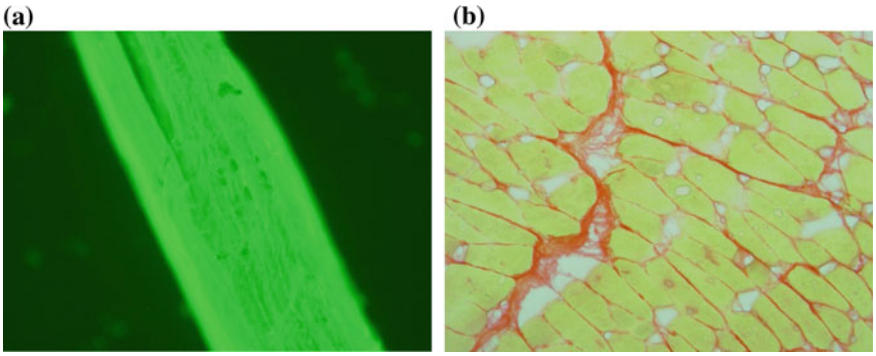


Fig. 14.1 Histology of extracellular matrix in connective tissues from the C57BL6 mouse. **a** Longitudinal section of a tail tendon, **b** cross section of the gastrocnemius muscle tissues

Figure 14.1a shows an image of a tendon under an optical microscope. Within the tendon are numerous lines depicting bundles of highly paralleled collagen fibrils which can only be seen under an electron microscope. When the tendon is loaded in tension, a high proportion of the fibrils will be recruited for force transmission. The direction of the force generated is along the axis of the tendon. Similar highly paralleled collagen fibrils are found in the ligament. In other connective tissues, e.g. muscles (Fig. 14.1b), the collagen fibrils may be less aligned; for muscles, apart from the muscle cells (i.e. fibre-like cells), the tissue also contain collagen fibrils (indicated by the region of red stain) which appears as thin mesh enclosing each muscle fibre-like cell (green in colour).

The proportion of the fibrils that can be easily recruited to carry the load from muscle to bone (in the case of tendons) or bone to bone (in the case of ligaments) depends on the orientation of the fibrils. These descriptions of the collagen fibrils and other ECM components apply to a great many soft connective tissues, including those of sea animals such as echinoderms, which is the subject of our discussion in this chapter.

With regards to the strategy for repairing damaged soft connective tissue in reconstructive surgical procedures, one important consideration is that the repair site must possess a structure that can support load [6]. From a mechanical engineering perspective, this means that such a repair material (known as the scaffold) would have to possess the desired structural and materials properties. In addition to this criterion are a range of other considerations, namely biocompatibility [7], such as immunological compatibility. It must be emphasized that given that there is still a lot of unknowns in the connective tissues, one would expect that the motivation to design and fabricate the material from scratch, such as the much-touted 3D printing approach to tissue engineering, would seem far-fetch. To this end, how can we acquire such a material for assisting tissue repair? One way is to manufacture biomaterials derived from extracts of biological tissues, such as chitosan from the exoskeleton of crus-

taceans [8, 9]. Another way is to harvest like-tissue from another species, followed by decellurizing the tissue to obtain the ECM [6].

There are a number of excellent tissue candidates that one can choose—namely porcine, bovine even human dermal tissues—that are now commercially available for obtaining the ECM for use as scaffold [6]. ECM-based biological scaffold materials from these intact mammalian tissues are applied in in tissue engineering as reported in preclinical studies and in clinical applications [10]. From the perspective of sustainable use of materials, interests in other sources are also being researched. Researchers are turning to marine sources such as echinoderms. These sea creatures are also able to provide collagen for producing ECM scaffold. The strategy is to separate the unwanted from the wanted (favourite edible) parts during harvesting. For instance, one may collect unwanted sea urchin soft connective tissues after the edible parts are removed [11–13].

The focus of this chapter is on the highly paralleled collagen fibrils in ECM of ligaments from the echinoderms. Our argument is developed from the ECM biology and stress transfer mechanisms for tendons (next section). Here, we present an examination of the nature of collagen fibrils, and the laying down of collagen fibrils within the proteoglycan-rich hydrated ECM. This is followed by an exploration of the structure-function implications in relation to a framework for the mechanisms of stress transfer, focussing on how taper in collagen fibrils, through the inter-play between key proteoglycans and collagen macromolecules, influence the tissue to take up stress, as the tissue is subjected to an increasing applied load until it ruptures apart. Then we reviewed how manufacturing methods may influence the structural and mechanical properties of the scaffolds and also highlighted the implications on tissue functionality during in vivo degradation and remodeling of ECM scaffolds [10]. In Sect. 14.3, we discussed the recent clinical applications of collageneous bioscaffold from the echinoderm sources.

14.2 Basis of Structural Support in Soft Connective Tissue

14.2.1 Overview

Echinoderms such as the sea urchin of the Paracentrotus lividus species is an economically important human food source [12]. This species may also be regarded as a potential source of collageneous connective tissue for use as scaffold materials in biomedical engineering. The tissues of particular interest are the compass depressor ligament and peristomial membrane, which are by-products of the food industry [12]. The normal practice is that these tissues are discarded together with the body parts, after the gonad is removed [12]. This section is intended to discuss the fundamentals relating to structure and mechanical properties of collageneous connective tissues that have lend to our understanding of how the echinoderm connective tissue (e.g.

compass depressor ligament and peristomial membrane) can be processed into useful biomaterials for making bioscaffolds for tissue engineering.

14.2.2 Structure of Collagen Fibril

Collagen are protein molecules with a basic structure comprising three tightly bound helical polypeptide chains, and an overall shape which resembles a rod with an estimated length of 300 nm [14]. Each polypeptide chain is formed from Gly-X-Y repeats [14]. These molecules aggregate to form various types of structures for supporting physiological loads. The majority of the collagens can be classified according to the nature of their aggregated forms. As of now, there are more than 30 types of collagens and a thorough reveal of collagen and fibrils has been reported recently [15]. (For the interest of the reader, conventionally, to reference the variety of collagen types, the vertebrate collagens are given a Roman numeral, e.g. type I collagen.)

With regards to the different collagen types, the majority of collagen found in fibrils are types I, II, III; the minor collagens are types V and XI [14]. Thus, we find type I collagen predominates in tendons; type II collagen predominates in cartilage [14]. Two or more types of collagen could predominate in certain tissues, e.g. type I and III collagen in the vascular system. Type III related collagen fibrils can be found as with small diameter in embryonic and vascular tissues [14]. Some collagens, namely types IX, XII and XIV, are bonded to fibril forming collagens specifically at the fibril surface. Type IX collagen is found on the surface of the type II collagen fibre; it could be covalently bound to type II in an anti-parallel manner. While not much is known about the mechanical function of type IX, it could possess good thermal stability [16].

The collagen molecules are axially configured, in a so-called ‘quarter-staggered’ (over-lapping) arrangement, and laterally dispersed in a radial pattern, within the collagen fibril [17–20]. The meridional and equatorial intensities of the x-ray diffraction pattern respectively contain important information about the axial and lateral arrangements of collagen molecules in fibrils.

In fact, analysis of the x-ray diffraction patterns has enable the revelation of the anisotropic property of fibrils from the Bragg peaks in the meridional and equatorial regions. From the meridional regions, while one may infer about the ‘D repeats’, characterised by light-dark bands (period = 67 nm) observable in electron micrographs, from the regular-spaced Bragg peaks (Fig. 14.2a), these peaks also yield information about the axial staggering of collagen molecules. Any change in the position of these peaks may implicate changes in the long-range axial crystallinity of the semi-crystalline structure of the fibril [21]; this property has been used to determine the deformation at molecular level when the fibril is deforming under tension [22].

The characteristic triplet of diffraction peaks occurs in the equatorial region and is observable at short camera length [18]; Fig. 14.2b shows a single blob because the pattern was acquired at a long camera length of 10.2 m (which corresponds to

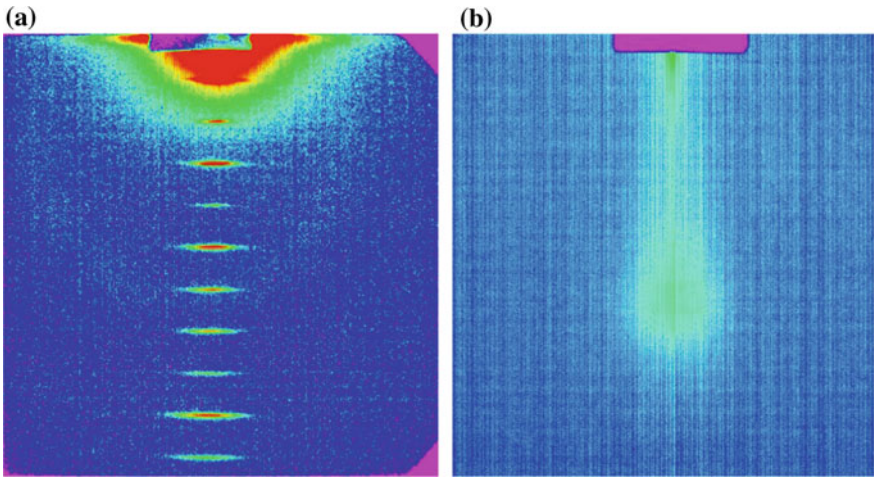
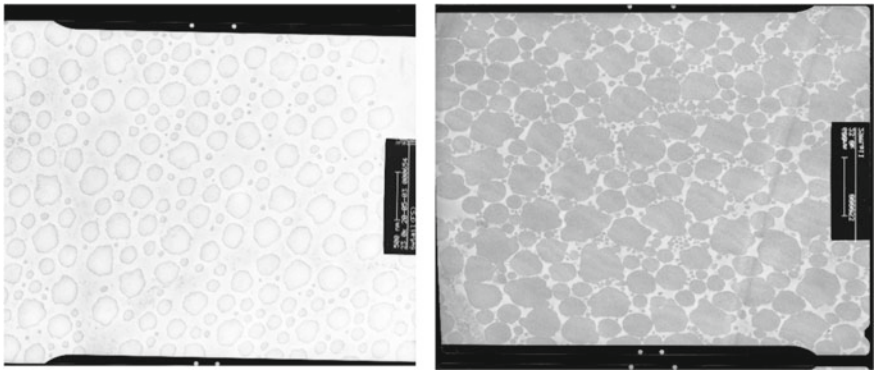


Fig. 14.2 X-ray patterns of collagen fibrils from tail tendons of C57BL6 mice. **a** The meridional peaks as revealed from small-angle x-ray scattering patterns. **b** The diffuse peak as shown in wide-angle x-ray diffraction patterns. These results were obtained using synchrotron x-rays (Beam-line 2.1, at Daresbury Laboratories, UK)

small angle scattering). It is possible to evaluate these peaks (Fig. 14.2b) using a model of the gap and overlap regions for the three-dimensional axial staggering of collagen molecules [17, 19] to derive important information about the packing of the collagen molecules in the radial direction. Such a model could help identify the precise changes in the radial packing influencing the Poisson ratio of these fibrils as demonstrated very recently in ageing studies by Haverkamp and co-workers [23].

While small angle x-ray scattering has been proposed as a viable method to study collagen fibril sizes [24], many studies prefer to rely on electron microscopy. Indeed, electron micrographs of the transverse section of connective tissues such as tendons have been used to study the sizes and dispersion of collagen fibrils in the PG-rich hydrated ECM (Fig. 14.3a). What is found commonly is that the cross section of fibrils is near-circular and non-uniform in sizes in young tissues [2, 25–27].

The fibril size effects on the mechanical properties of the tissue has been a subject for debate until 2012 when Goh and co-worker established a detailed analysis of the connection of the different size distribution of collagen fibrils to the resilience and fracture toughness of the tendons [26]. The origin of these sizes is a subject for speculation: the small fibril size may be associated with short fibrils, and may have been formed shortly while the large fibril size may be the result of further fibril self-assembly after extrusion from the fibroblast, as well as fusion of two or more fibrils post fibrilogenesis [2, 25–27]. On the other hand, if we consider that these are tapered fibrils, then the different sizes could be attributed to the sectioning of fibrils at different points [27]. Additionally, there are difficulties arising from the serial sectioning of fibrils: (1) there is limited information concerning whether the fibrils seen so far possess lengths that span the entire tissue, (2) is the small size fibril



(a) 11 WEEKS OLD **(b) 29 MONTHS OLD**

Fig. 14.3 Cross-sections of tail tendons in young (11 weeks old) and old (29 months old) C57BL6 mice. Images (unpublished) were provided by David F. Holmes. Similar patterns may be found elsewhere in an earlier publication [25]

cross-section a part of a longer fibril with larger fibril diameter at the fibril centre but not observable by this method [27].

Figure 14.3b shows the increasing irregularity of the morphology of the fibril cross section with age; no appreciable increase with age was observed for the packing of fibrils. Quite a while back, it is believed that decorin proteoglycans are capable of regulating the size and morphology of collagen fibrils [28]. There is a high similarity in the irregularity observed in tissues from older animals with those from knock-out mice deficient in the gene for decorin [28].

It has been proposed that the irregularity is due to fibril tip to shaft fusion or fibril shaft to shaft fusion [29] but whether this could be caused by a decrease in the decorin density on the fibrils remains to be tested. Decorin is a member of the family of small leucine repeats PGs (SLRPs) that includes fibromodulin and lumican [30]. Decorin binds to procollagen [31] and evidence of surface-bound decorin proteoglycans at the ends of collagen fibrils led to speculations that decorin might have an important role in the fibrillogenesis process because the collagen may be able to accommodate the proteoglycan during fibrillogenesis [29].

Although some investigators have shown that there are no changes in the mechanical properties with the removal of the decorin proteoglycans [32], Soslowsky and co-workers have recently shown that decorin contribute to tendon's response to load based on studies using decorin knockout mice [33]. Decorin-null tendons revealed significant changes in the mechanical properties across different age groups, accompanied by location-dependent collagen fibre re-alignment changes, suggesting a site-specific role for these molecules in loading [33]. That decorin proteoglycans could result in reduced magnitudes in the mechanical parameters as they are associated with the interfibrillar matrix is still an ongoing topic for debate.

It is an attractive theory because of the following findings: (1) Optical tweezer mechanics study of decorin-decorin interaction shows that the interaction may be disrupted at a force of order of 10 pN [34]. The low force suggests that the interaction is not contributed by covalent bonding. As ECM occupies the bulk of the tissue, the non-covalent, and presumably reversible, forces between these proteoglycans could be an important contributory factor to the structural and mechanical integrity of the connective tissues; (2) Evidence of decorin proteoglycans on the surface of collagen fibrils [35, 36]. The proteoglycan can in turn modulate fibril size during fibrillogenesis [30]. It then follows that absence of these molecules could have a profound effect on the fibril size; transgenic mice which are null for decorin show abnormal collagen fibril diameters [30].

ECM is a hierarchical architecture [2, 27]. At the length scale of the fibril, the fibril is made up of microfibrils [19]. At a larger length scale, the collagen fibrils bundle up to form collagen fibres. At the next higher length scale, the collagen fibres bundle to form fascicles.

14.2.3 Mechanism of Reinforcement

When connective tissues, such as tendon and ligaments, are subjected to an increasing tensile load the mechanism of stress up-take in the tissue varies throughout the loading process until the tissue ruptures [2, 4, 27].

How the tissue takes up load finds an analogy to how fibre reinforced composites can withstand loads that tend to pull them apart [5]. In this case, the collagen fibrils are responsible for reinforcing the weak PG-dominated hydrated interfibrillar matrix [4, 37]. Collagen fibrils are long and slender structures that taper (somewhat paraboloidal) to each end, as observed under a transmission electron microscope [38, 39].

It has been shown using atomic force microscopy that collagen molecules possess a rupture force of the order of 1000 pN [40]. Taking the energy to rupture to be equal to the displacement at rupture multiply to the force to rupture, one finds that the energy to rupture is of order of 1×10^{-4} pNm. Clearly the mechanical properties of collagen fibrils are remarkable but how does the fibril provide reinforcement to the ECM? The biomechanical properties of the tissue, namely fracture strength and modulus of elasticity, may be said to depend on the collagen fibril volume fraction V_f and the material properties of the fibrils, according to the rule of mixtures for fracture strength and modulus of elasticity that are often applied for studying engineering fibre composites [5]. The fibril volume fraction is a parameter which provide an indication of the number of fibrils present in ECM but could also accounts for the inter-fibrillar distance which we will see in the following section. However, these do not help explain how collagen fibrils provide reinforcement to the ECM.

Over the last ten years, using concepts applied to engineering discontinuous fibre reinforced composites [3, 41–45], Goh and co-workers have laid down the key foundations for the reinforcement mechanisms that relate to how collagen fibrils take up

stress, at different stages of the loading process [2, 4, 27, 37, 46]. These mechanisms are the elastic stress transfer, plastic stress transfer, and rupture [2, 4, 27, 46]. This section will discuss the key concepts underpinning these mechanisms that have helped shaped our insights for collagen fibril reinforcing connective tissue.

We begin with the concept of elastic stress transfer mechanism, which regulates the initial loading stage [4]. Here, the proteoglycan-rich matrix deforms elastically. At the interface between the collagen fibril and the proteoglycan-rich matrix, the interactions of decorin proteoglycans on the fibril with macromolecules in the PG matrix and with decorin PGs on adjacent fibrils generate interfacial shear stresses which place the fibre in tension as it stretches elastically.

If the fibril were paraboloidal in shape at the ends [38], the stress would be a minimum at the fibril centre and increases and peaks near the ends of the fibril. If the fibril were uniform cylindrical in shape, the stress would peak at the centre of the fibril and decreases non-linearly to zero at the fibril end [4]. Concentrating stresses near the fibril end makes good sense because should a portion near the end breaks, the effectiveness of the bulk of this fibril for reinforcement would not be appreciably compromised. This would not be the case should stresses be concentrated at the fibril centre; the fibril would suffer a significant lost in length should the fibril break at the fibre centre.

Unfortunately, while it is certainly more advantageous for a fibril to be paraboloidal in shape at its ends than uniform cylindrical, the question of whether the fibril is uniform cylindrical or paraboloidal in shape is still debatable because the only people who have witnessed the latter shape are Holmes and co-workers. Another point to note here is the parameter known as the relative stiffness of the fibril to the proteoglycan-rich matrix, E_f/E_m. In all the four cases shown here in Fig. 14.4, it is observed that high E_f/E_m corresponds to high stress uptake in the fibril; on the other hand, low E_f/E_m corresponds to low stress uptake in the fibril. What this mean is that while high E_f/E_m bestows the fibril with the capacity to take up high stress, providing good stress transfer from the proteoglycan-rich matrix to the fibril, under an increasing applied load, this mean the stress in the fibril will increase and if it eventually reaches the fracture stress of the fibril, the fibril breaks. On the other hand, low E_f/E_m reduces the capacity of the fibril to take up high stress; this means that the stress transfer mechanism is not as effective as when the E_f/E_m is high. Thus, when E_f/E_m is low, a part of the load is taken up in the proteoglycan-rich matrix and the matrix will readily disrupt at a lower applied load as compared to the case when the E_f/E_m is high.

Between the stages regulated by the elastic and plastic stress transfer mechanisms, there is an intermediate stage in which the fibril undergoes one or more of the three modes of stress transfer, namely Mode α, β, and χ. Mode α is said to occur when the deforming proteoglycan-rich matrix yields and turns plastic adjacent to the fibril-matrix interface. In the case of uniform cylindrical fibrils, stress concentrates in the proteoglycan-rich matrix around the end and this may lead to matrix yielding. For tapered fibrils the lower stress concentrations in the proteoglycan-rich matrix around the ends may make them less susceptible to Mode α. Mode χ is said to occur when a crack at the debonded fibril end propagates into the proteoglycan-rich matrix but

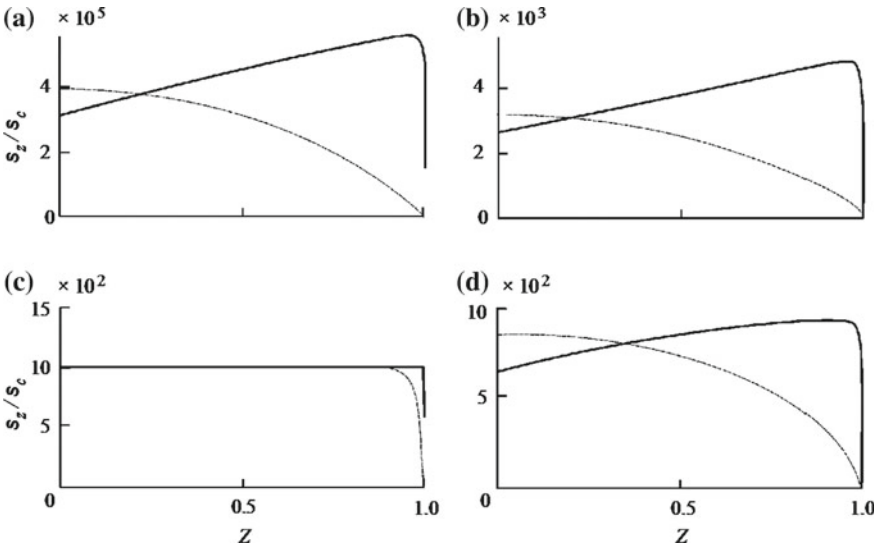


Fig. 14.4 Distributions of normal stresses, σ_z, versus distance along the collagen fibril axis, Z, during elastic stress transfer. **a** High E_f/E_g and high q. **b** High E_f/E_g and low q. **c** Low E_f/E_g and high q. **d** Low *Ef/Eg* and low q [4]

not along the fibril-matrix interface. There is some similarity between Mode χ and the engineering concept of mode I crack, i.e. parting of two surfaces; in this case both concepts suggest that stress transfer will not occur across the crack planes and subsequently reduces the effectiveness of stress transfer between the proteoglycan-rich matrix and fibril.

Mode β is said to occur when a crack initiates in the interface at the debonded fibril end and propagates along the interface. Frictional stress transfer occurs as the deforming proteoglycan-rich matrix slides over the fibril surface. The rate of debonding is related to the relative stiffness of the fibril to the proteoglycan-rich matrix, E_f/E_m; high E_f/E_m means that the fibril can take up higher stress but that also mean that if the applied load increases to a high level, this translate to a high stress in the fibril which could exceed the fracture stress of the fibril and hence induces fracture. In other words, the greater the elastic mismatch between the fibril and proteoglycan-rich matrix, the higher the rate of fibril fracture. On the other hand, low E_f/E_m means that the fibril has a lower stress uptake capacity; as less stress can be transferred to the fibril, the rest is bore by the proteoglycan-rich matrix and this will readily induce failure in the matrix at a lower applied load.

The failure may appear in the form of cracks or debonding; in the case of the latter, shear-sliding action begins when the interfacial shear stress overcomes the frictional stress and a cohesive sliding resistance. When sliding occurs between the fibril and the surrounding matrix, the value of the interfacial shear stress is constant throughout the interface. There is some similarity between Mode β and the engineering concept

of mode II crack, i.e. shear failure; in both cases, it suggests that stress transfer via friction at the crack surfaces. A modified Rice and Tracey micro-void nucleation, growth and coalescence model has been used to predict the crack propagation in a finite-element analysis, leading to insights concerning how a proteoglycan-rich matrix crack is formed and how the crack propagates in the presence of voids in the region ahead of the crack which nucleate, grow and coalesce due to the presence of high stress at the fibril corner. A critical fracture strain may be defined as a function of void size and the level of the stress magnitude around the region; when the stress at a point in the proteoglycan-rich matrix was greater than this critical fracture strain, this results in a crack.

After the intermediate stage, as the applied load increase, the reinforcement of the ECM by the collagen fibrils is regulated by the plastic stress transfer mechanism. This mechanism involves the disruption of the interfacial bonds between the collagen fibrils and the PG-rich matrix. The disruption cuts across the entire interface, initiating at the fibril end, propagating towards the fibril centre and ends at the fibril centre. The proteoglycan-rich matrix now deforms plastically and shear-slides over the fibril surface. The plastic stress transfer mechanism is essentially a simultaneous occurrence of mode β and α at the interface, and in the adjoining proteoglycan-rich matrix region, respectively.

Goh and co-workers have evaluated a finite element model consisting of an elastic fibre in tension under the application of a constant shear stress on its surface to study plastic stress transfer [41]. If the fibril were uniform cylindrical in shape, the axial stress peaks at the centre of the fibril and decreases linearly to zero at the fibril end (Fig. 14.5). If the fibril were paraboloidal in shape at the ends, the axial stress also peaks at the fibril but decreases non-linearly to zero at the fibril end (Fig. 14.5).

During the process of deformation, stress transfer from the proteoglycan-rich matrix to a fibril may cause the fibre to break at fibril centre where the stress reaches the fracture stress. This results in fibril fragments. Further fragmentation process may

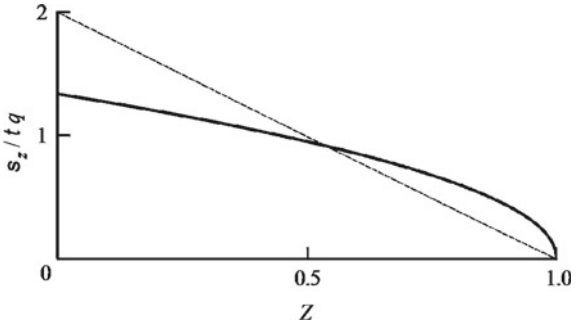


Fig. 14.5 Distributions of normal stresses, σ_z, versus distance along the collagen fibril axis, Z, during plastic stress transfer. Dark bold line refers to paraboloidal shape; light thin line refers to uniform cylindrical shape. Symbols τ and q denote fibril-matrix interfacial shear stress and fibril aspect ratio (a parameter for the slenderness of the fibril)

occur, but this would eventually stop when the fragments are too short. This leads to the idea of a critical length parameter for the collagen fibril; this is the length of the fibril below which the stress uptake into the fibril would not be sufficient to reach the fracture stress and the fibril would not fracture. Although one could estimate the length of collagen fibrils in tissues, e.g. on the basis of the number of fibrils ends observed in a cross section [47], no attempts has been made to measure the critical length of collagen fibril experimentally.

Finally, beyond plastic stress transfer, at higher applied loads, the tissue begins to fail by a variety of mechanisms: debonding at the fibril proteoglycan-rich matrix interface, fibril fragmentation, cracking in the proteoglycan-rich matrix and fibril pull-out. The order and importance of these events is immaterial, but it is important to note that the variety of failures have been well-observed in scanning electron micrographs in other studies such as thermal effects and ultraviolet irradiation effects [48, 49]. Fibril pull-out may occur when fibrils are drawn out from the crack faces within proteoglycan-rich matrix. This may be initiated by the combination of Modes β and χ. If Mode β did not occur, the proteoglycan-rich matrix crack arising from Mode χ may propagate to neighbouring fibrils; these neighbouring fibrils become responsible for bridging cracks in the proteoglycan-rich matrix. In both cases, fibril pull-out may occur when the fibril is unable to bridge the crack. The ability of a fibril to bridge a proteoglycan-rich matrix crack depends on the interface, the fibril strength and fibril modulus. If bonding is present at the interface, then the trigger for interfacial failure depends on the yield stress related to the interfacial shear; if debonding has occurred, then the degree of pull-out depends on friction as a result of sliding. There are two possible outcomes of fibril pull-out while bridging a proteoglycan-rich matrix crack. The first is that if the crack to propagate were to be deflected by neighbouring fibrils, the crack could propagate along the interface instead (Mode β). The second outcome is that the fibril could fracture when the applied load increases so that attempts to bridge the crack results in high stress uptake in the fibril beyond the level of the fibril fracture stress.

14.3 How Processing Bioscaffolds Affects the Scaffold Structural/Mechanical Integrity

14.3.1 Overview

The approach to treat the connective tissue of the echinoderms to derive the ECM scaffold material involves physical/mechanical and biochemical processes. The processing steps typically involve decellularization, hydration, dehydration, powdering and gelation, with disinfection during the process (Fig. 14.6). Each of these steps may affect the structural/mechanical integrity of the bioscaffold (as well as the type of host response) that which it is intended for. Here we shall focus on the first three steps to illuminate the effects on the mechanical integrity of the scaffold.

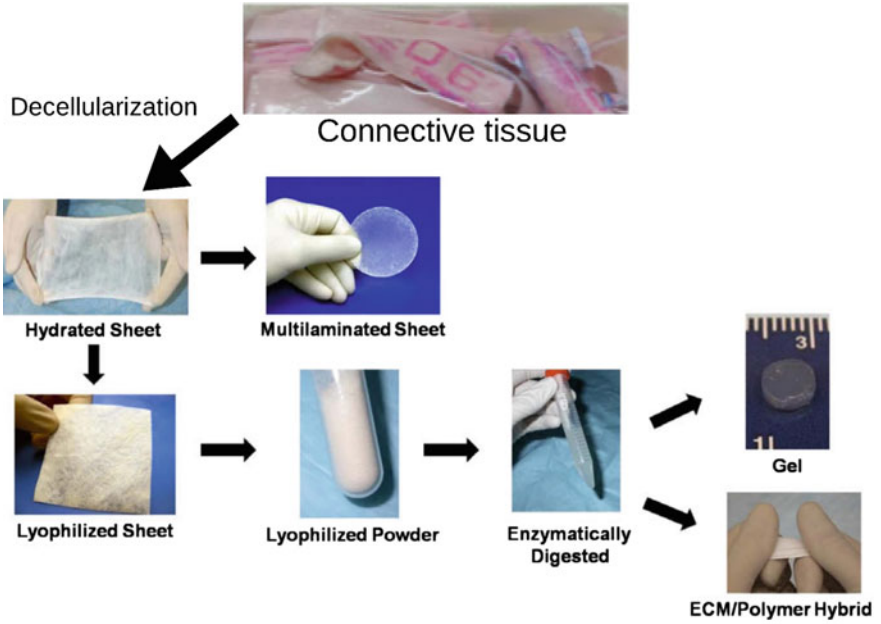


Fig. 14.6 Schematic of the processing of extracellular matrix (ECM) scaffolds from connective tissue. After the tissue is harvested, it undergoes decellularization to obtain a hydrated bioscaffold. Three-dimensional scaffold may be obtained by vacuum-pressing several such hydrated sheets to form a laminate scaffold. Lyophilization of the hydrated sheets, followed by a powdering process, may be implemented. The power may be enzymatically digested into a liquid; further processing by repolymerization or blending into a synthetic polymer then yields the ECM/polymer scaffold. Reprinted from [10], with permission from Elsevier

14.3.2 Decellularization

The aim of decellularization is to remove cellular material in the tissue so that what remains is the ECM [50]. This is important because cellular material may cause immunologic response in the host body; further discussion is found in the last paragraph of this section. Ideally, the method must not affect the ECM composition and structural/mechanical integrity (as well as biological activity). The strategy to remove cells is concerned about the removal of cell contents. To this end, one may target the breakup of the cell membrane by sonication, agitation and freezing/thawing [51]. As the membrane burst, cell contents are released but only further rinsing of the tissue may remove the cell remnants from the ECM [51].

Ideally, any decellularization process should not alter the structural/mechanical properties and biological properties of ECM [51]. In practice, this is not the case. One finds that fibrils are broken up during decellurization; the fibril-matrix interface and proteoglycan-rich matrix are disrupted. Detergents ae used in the decellularization process but while some detergents may be disruptive to ECM collagen in some

tissues, the same type may have minimal disruption to other tissues [51]. Additionally, glycosaminoglycans (GAGs), which holds water in ECM, may also be removed from the scaffold during decellularization [51]. Unfortunately, GAGs play an important role in regulating the viscoelasticity of the tissue and the absence of GAGs may result in diminution of the viscoelastic properties of the tissue [51]. Clearly it is important to develop effective methods for decellularization without drastically affecting the mechanical properties of the tissue [51].

In practice, decellularization methods may not be able to completely remove all cellular DNA-related materials [51]. Adverse immunologic response by allogeneic and xenogeneic recipients of the ECM scaffold material follows when antigenic epitopes found in the cell membranes and intracellular components of tissues and organs are not removed [51]. This is because xenogeneic and allogeneic cellular antigens are respectively recognized as foreign by the allogeneic and xenogeneic host [51]. The immunologic response may be inflammation or overt immune-mediated rejection [51]. Of note, it is worth noting that many molecules that are native to the extracellular matrix are well conserved across species and that they pose a lower risk of immunologic response in xenogeneic recipients [51].

14.3.3 Hydration

For the ECM scaffold it is important to maintain hydration within the scaffold in order to ensure that the structural/mechanical integrity are not altered [51]. Implementing hydration to recover the structural/mechanical integrity after subjecting to possible dehydration (during decellularization and sterilization) assumes that the dehydration/hydration is reversible, but this is not necessarily true [51]. When the ECM is dehydrated, the collagen fibrils are drawn closer to one another and this could fracture the fibril in the absence of a hydrated matrix which provides a buffer between the fibrils [52].

It has been pointed out that bioscaffolds that remain hydrated throughout the decellularization and sterilization process are better able to facilitate cellular attachment (and cell infiltration) than bioscaffolds that are subjected to a dehydration step followed by rehydration [51] but the key reason could be that the fibrils, being more intact in the former, allows for inter-twinning which lends to a more mechanically stable network of fibrils in the ECM. However, hydration comes with a price: it is said that the bioscaffold would leach off soluble growth factors (such as VEGF and b-FGF) present in the material during storage [51].

14.3.4 Dehydration

There are two advantages of having a dehydrated bioscaffold. First, dehydrated bioscaffolds are easier to handle (e.g. for vacuum pressing to create laminates) dur-

ing processing; second, dehydrated bioscaffolds can also minimised leaching/loss of growth factors during storage [51]. Dehydration can be carried out by lyophilization, followed by sterilization [51]. Lyophilization is a freeze drying (low temperature, low pressure) process by which the water is removed from the bioscaffold by sublimation.

While lyophilization can be used to preserve the ECM of biological graft tissues from degradation during storage, but at the expense of modifying the collagen fibril ultrastructure [51]. In particular, as the thickness of the bioscaffolds may be decreased following lyophilization (typically by 30%) because the water phase is removed, this results in a collagen ultrastructure that features a more tightly packed fibrils. Additionally, as the proteoglycan-rich matrix is now greatly reduced in volume fraction because the water phase is removed, this means that there is a significant disruption to the interaction between proteoglycans and proteoglycan and collagen. These changes may be irreversible when water is reintroduced: (1) the more compact fibril packing will not make it easy for the bioscaffold to recover the original water phase, and (2) the disruption of the interaction between the proteoglycans and collagen at the fibril-matrix interface means that the effectiveness of fibril stress uptake is reduced as it can only occur via the shear-sliding action, regulated by either Mode β or plastic stress transfer mechanisms [2, 27].

Vacuum pressing process is intended to produce a 3D enhanced-anisotropic structure by tightly stacking layers of thin bioscaffolds. This is analogous to the stacking of layers of unidirectional continuous carbon fibres (where fibres in consequentive layers are in different directions) to form a carbon fibre reinforced polymer composite (CFRP) for structural application. This is also analogous to the anisotropic collagen ultrastructure in skin dermis [53]. In all cases, the laminate results in enhanced mechanical properties, such as higher strength and stiffness as compared to the individual layer. More importantly, it is intended to mimic the structural/mechanical environment of the specific tissue in which the implant would be introduced for, e.g. tissue repair [51]. However, such a vacuum-pressed bioscaffold can also exhibits reduced extensibility, as compared to the individual layer [52]. Thus it is important to develop an optimal configuration and method of processing of an ECM scaffold for the intended clinical application.

14.4 Potential Clinical Applications

So far, the commercially available and FDA-approved biological scaffold materials are made from human skin, human fascia lata, human dermis, cadaveric human dermis, porcine small intestinal submucosa, horse pericardium, fetal bovine skin, cadaveric fascia lata, in dry or hydrated forms [10]. While there are several reports highlighting the possible use of echinoderm connective tissues, such as sea urchin ligaments and membranes, as biomaterials for constructing bioscaffolds for tissue engineering and other biomedical applications, these marine-related bioscaffolds have not appear in the market yet. This section is intended to assess some of the possibilities proposed by the researchers, in the context of mechanical viability.

The most commonly suggested area of application for the echinoderm connective tissue is for tissue repair. The processing of bioscaffolds from connective tissue has been discussed in Sect. 14.3. One group has already embarked on a long-term study (known as the MIMESIS project) to develop and commercialise collagen films made from sea urchin connective tissues [54]. Collagen extraction was performed according to the protocol of Matsumura [55] with additional modifications established by the group [12, 56]. They have claimed that their method could also extract intact collagen fibrils; their aim is to be able to process a bioscaffold with the appropriate hierarchical architecture as that found in the host tissue where the bioscaffold would be implanted. In the report, they have also carried out test on their bioscaffold using mammalian cells [11]. More specifically, it has been suggested that the decellularized material may be used to treat connective tissue pathologies characterized by alteration of the viscoelastic properties [57]. This could be implemented by implanting the treated decellularized material into the pathological site. The mechanical properties of the implanted material would have to be of similar viscoelastic properties as that of the host tissue. To be able to address this effectively, the implanted material would have to be treated to an appropriate level of hydration over a given duration. Recall that if the hydration is not adequately achieved, this would result in a high E_f/E_m which is fine if the fibrils in the ECM can undergo elastic stress transfer as a high E_f/E_m means that the fibrils are able to take up high stress during elastic loading via the shear-lag action. But if the inadequate hydration results in poor interaction between the proteoglycan-rich matrix and the fibrils, when the implanted material is loaded, the fibrils would only be able to undergo plastic stress transfer, which is not ideal as stress transfer via the shear-sliding action is not as effective as via the shear-lag action.

The mutability of the echinoderm connective tissues has been well-studied and it has been suggested that this unique property can be exploited to develop collagen barrier-membranes for Guided Tissue Regeneration (GTR) [13]. Of note, one can now find commercially available membranes for GTR or soluble/reassembled (fibrillar) bovine collagen substrates but alternative sources from marine animals have yet to be commercialised. Like the bovine sources, use of the tissue from sea urchins is sensible in terms of eco-sustainability as this addresses the recycling tissues from food wastes. Ferrario and co-workers have used the mutable connective tissues (MCTs) from different echinoderm models (sea urchin, starfish and sea cucumber) to produce echinoderm-derived collagen membranes (EDCMs). These EDCMs could result in similar rate of cell (e.g. fibroblasts) proliferation and cell morphology as those from commercially available bovine collagen substrates.

The mutability of the MCT has inspired Trotter and co-workers [56] to propose a ‘hybrid’ biomaterial inspired by the MCT. Such a hybrid biomaterial could compose of synthetic interfibrillar matrix and collagen fibrils of sea cucumber dermis origins [56]. Clinically speaking, MCT-like biomaterials could be useful for tissue engineering of soft tissues, i.e. for application in regenerating tissues in a dynamic environment [58]. This concept is still a novelty but deserves further consideration in the near future.

Finally, the echinoderm connective tissue that exploits the destiffening property could have useful application in the cosmetic industry [57]. Cosmetically speaking, the decellularized ECM could provide anti-aging therapy for destiffening the aged skin.

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Chapter 15
Clinical Application of Biomimetic Marine-Derived Materials for Tissue Engineering

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Abstract The use of advance technology allocated a scientific community with significant development in the field of tissue engineering and medical sciences. Developing a biomaterial to replace the diseased or damaged tissue is a paramount importance for an effective regenerative approach, so that the original structural and functional status is recovered. Due to its rich biodiversity, marine environment yields immense potential and offer various organisms from which promising natural substances can be isolated to mimic the tissue ECM (extracellular matrix) in the body. Findings by various researchers both in vitro and in vivo also support the opinion that the derived structures from aquatic origin have optimistic potential for biomedical application. In this chapter, we shall discuss some of the marine-derived biomaterials which can be employed for various tissue engineering approaches. Marine ecosystem nourished a wide variety of creatures like corals, seashells and sea urchins from which various biopolymers can be extracted. These bio-molecules offer a new dimension for clinical application in dentistry, oral and maxillofacial surgery, wound healing, local drug delivery system, cartilage and bone tissue engineering. As the substances derived from marine origin are organic in nature, they are usually non-toxic, bio-

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compatible, bioactive and well tolerated by the body, which boost their efficacy for tissue engineering application.

Keywords Marine biomaterials · Tissue engineering · Chitin and chitosan · Alginate · Calcium carbonate and hydroxyapatite · Collagen · Biosilica · Fucoidan · Carrageenans · Glycosaminoglycans · Chondroitin sulphate · Hyaluronic acid

15.1 Introduction

Transplantation of organ and reconstructive procedure have gained considerable interest in advanced medical treatment to save lives, but occasionally may be coupled with some limitations. Usually, these approaches require either organ donation from others or tissue transplantation from a second surgical site and are associated with some complications like paucity of donor organs for the first, while the latter is associated with pain and morbidity of the patient. These consequences need to develop alternate organs, tissues, and synthetic materials ex situ for application in the field of tissue engineering [1–3]. Tissue engineering thus appears to be an encouraging field with appropriate technologies to regenerate and restore the defect or diseased part of tissues or organs [4]. The existing tissue engineering strategies may include cell-based therapies to repair new tissues [5, 6] and application of nonliving biomaterial to support cell growth and differentiation [7].

For a successful implantation, the ideal biomaterial must exhibit specific properties like non-toxicity, elicit appropriate host tissue reaction, biological inert or corrosion resistance, no side effect and no adverse tissue reactions or rejections. Additionally, it should possess sufficient amount of mechanical and rheological strength. In the 21st century, biomaterial industry is one of the most rapidly emerging industry and accounts for 2–3% of the overall health expenses in developed countries [8]. During these periods, a wide variety of materials such as metals and its alloys, collagen, carbon-based materials, polymers, ceramics, and some composites structures combined with growth factors and stem cells have been studied with various degree of success [9, 10]. Their benefits are not without some drawbacks, especially the high costs of production [10, 11]. To overcome these issues, natural products emerges to be an ideal candidate since they are organic in nature, more biocompatible and higher bioactivity for cellular attachment and growth [12]. In addition to their application in bone tissue engineering, several biomaterials are also applied for soft tissue healing and furthermore, the present focus of both clinical and preclinical work on biomaterial is centered on ‘targeted drug delivery system’.

For instance, marine biotechnology allows application of scientific and engineering know-how to explore the oceans and develop biomaterials from aquatic origin like plants and animals [13]. Deep ocean exploration plays an important role in the development of biomaterials, health care services, diagnostics, as marine biotechnology helps in producing pharmaceutical drugs, chemical products, enzymes and other

industrial products which is essential for human well-being. It is well known that approximately 70% of the earth's surface is water, which contributes 90–95% of the biosphere by volume of living organisms and thus providing a wide range of biodiversity. During the past decades, with better knowledge of complex biomolecules and application of new tools in biotechnology, advanced and extraordinary new marine biomedical products have been obtained [14]. This exciting achievement in understanding the fundamental features of aquatic life has provided excellent opportunities to develop the new marine biomaterials. Advancement in marine biotechnology is a fundamental part of the economy in countries like USA, Japan, China, Korea, and Russia as well as other European Community [15, 16]. The application of marine biotechnology will provide an answer whether resources from marine ecosystem can be explored for human benefit and development of fundamental biological field as a whole [17].

The marine surroundings are abundant with various organisms which contain porous architecture, some of which are already being used as bone graft substitute, but others are still at its infancy [18]. For example, chitosan is a natural derivative of mucopolysaccharides called chitin, a biopolymer found in the exoskeletons of marine crustacean like shrimp, crabs, lobster, and other shellfish. Due to its biocompatibility, chitosan is an effective material to repair bone defects or damage part of bone. A product obtain from marine coral exoskeleton of *Porites* sp. can be fabricated into a scaffolding material for bone regeneration due to its properties like interconnected porous structural design, high compressive breaking stress, good biocompatibility, and resorbability. Examples of other promising marine products include collagens from jellyfish [19], polymers from marine Diatoms [20], chitin from marine sponges [21] and hydroxyapatite and calcium phosphates from fish bone and other organisms [22]. Various studies also aimed at effectively growing corals and marine sponges in natural habitats for commercial activities and biomedical purposes. Attempts have been made to farm marine sponges with a purpose of producing bioactive metabolites in practical amounts [23]. Natural materials like cuttlefish bone, sea urchin spines, and seashells have all been studied and investigated extensively as possible structures for bone tissue engineering application [18]. Accordingly, this chapter discusses on the clinical application of different biomimetic marine-derived materials for tissue engineering approaches.

15.2 Commonly Available Marine Biomaterials in Tissue Engineering Application

15.2.1 Chitin and Chitosan

Chitin, a natural mucopolysaccharides composed by a linear chain of β (1 → 4) linked N-acetyl-D-glucosamine units is found in fungi, diatoms, nematodes, arthropods, shrimps, crabs, lobsters, krill, and squid [24–26]. The chitinase enzyme can easily

degraded this linkage [27]. However, more attention needs to be paid to the deacetylated derivative of chitin i.e., chitosan. Chitosan is also a naturally occurring polysaccharide and is composed of D-glucosamine (70–90%) and *N*-acetyl-D-glucosamine (10–30%) units, connected by β (1 → 4) glycosidic linkage [27], found in crab, shrimp, lobster, coral, jellyfish, butterfly, ladybug, mushroom, and fungi. However, marine crustacean shells are the primary sources in the production of chitosan [28, 29]. Chitin and chitosan are known to be one of the major sources of pollution in the coastal areas of tropical and subtropical water, particularly in the Pacific, Atlantic, and Indian oceans as crab and shrimp are important marine organism with immense commercial value. Since it is biologically reproducible, biodegradable, environmentally non-polluting, biocompatible, nontoxic, and biologically functional, the natural chitosan is a flexible material for various biological and biomedical applications [30].

15.2.2 Alginate

Alginate is a natural marine biopolymer, widely distributed in the cell wall of seaweed and commonly extracted from brown algae (*Phaeophyceae*) including *Laminaria hyperborea*, *Laminaria digitata*, *Laminaria japonica*, *Ascophyllum nodosum*, and *Macrocystis pyrifer* by treatment with aqueous alkali solutions (NaOH) [31, 32]. Quantitatively, it is the main polysaccharide found in brown algae, contributing up to 45% of the dry weight and is accountable for its flexibility, its mechanical and structural functions as well as ionic exchange roles. Alginate is an unbranched anionic copolymer composed of both (1 → 4) linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) residues [32]. This guluronic acid (G) and mannuronic acid (M) block possess the characteristics of biocompatibility, non-toxicity, non-immunogenicity, and biodegradability. In fact, alginate is one of the most promising marine biomaterials with versatile applications in biomedical engineering due to its unique properties of gelation and biocompatibility. Alginate and its products can deliver many new biomaterials, in the field of cell immobilization, tissue engineering, wound healing and drug delivery. The remarkable structural mimicking property with extracellular matrices (ECM) of the tissues has led to more extensive use of alginate hydrogels for several biomedical applications [31, 33].

15.2.3 Calcium Carbonate and Hydroxyapatite

Hydroxyapatite (HAp) has a special contribution in the field of biomedical and biomaterial science because its mineral constituents are similar with the natural of bones. Calcium carbonate ($CaCO_3$), found in many marine organisms is not as appealing as calcium phosphates in terms of biomedical application, but may be a pioneer material for getting some of calcium phosphates compound like HAp [34, 35]. Though there are many desired characteristics, yet the unique properties like porosity, architecture,

pore interconnectivity etc. made them an ideal candidate for orthopedics and dental applications [36]. Hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$], a naturally occurring mineral of calcium apatite is derived from various marine sources, such as fish bone [37, 38]. Significant attention has been paid to fish bones and other marine waste for the production of HAp, owing to its capability in diminution of environmental pollution and leads to value-added production. They are also predominantly safe, biocompatible, and osteoconductive; which boost the suitability for application as bone graft alternate filler [39, 40]. As a result, HAp has been considered to play a vital role in a variety of bone-related applications [41–43].

15.2.4 Collagen

Vertebrates are a rich source of collagen protein. Collagen is a structural matrix for bones, skin, muscle, and cartilage which organized in a fibrillar arrangement. Fish is one of the foremost sources of marine collagen which also mimic the same quality obtained from the other source. It's application ranges from cosmeceuticals, nutraceuticals, pharmaceuticals, functional food, to biomedical arena, especially bone-related diseases [44]. In general, bovine collagens lead to possible disease transmission like encephalopathy or transmissible spongiform encephalopathy. Thus, marine source received considerable attention due to its added benefit of lack of disease transmission. Marine sources of collagen include marine sponge *Chondrosia reniformis Nardo* [45]; rhizostomous jellyfish [46]; fish waste materials, skin, bone, and fins [47]; jellyfish [48]; paper nautilus [49]; muscles and skins of marine animals [50]; cuttlefish [51]; squid skins [52]; marine sponge [53]; and *Sebastes mentella* [52].

15.2.5 Biosilica

Biogenic silica, also referred to as Opal, is commonly recognized as biosilica that consists of glassy amorphous silica and is widely distributed in various aquatic organisms such as sponges, diatoms, radiolarians, and choanoflagellates [54]. Among all these organisms', sponges and diatoms are the two most important sources of biosilica [55]. Apart from the above sources of marine collagen, some sponge species also contributes as a source of biosilica and in particularly the two common classes of sponge which possess a silica skeleton: demospongiae and hexactinellida. The axial filament of the sponges consists primarily of the silicate in which converts to biosilica by silicification [56]. It has been identified that the naturally occurring silica has a desirable property as a biocomposite with high flexibility and toughness [57]. In addition, silica-based biomaterials have properties of biocompatibility, bioactivity and expedient reaction product after implantation [58]. So, the silica based bioactive glasses possess a wide range of biomedical applications including bone tissue

replacement, soft tissue augmentation, maxillofacial reconstruction, urological tissue augmentation, ossicle replacement etc.

15.2.6 Fucoidan

Fucoidan (a sulfated polysaccharide) is one of the most important marine biomaterials commonly found in brown algae such as mozuku, komby, limumoui, bladderwrack, wakame, hijiki, and sea cucumber. Fucoidans are also obtained from edible marine species such as *Fucus vesiculosis*, *Cladosiphon okamuranus*, *Laminaria japonica*, and *Undaria pinnatifida*. Structurally, the main skeleton of fucoidans consists of α-1,3-linked sulphated l-fucose; a repeating sequence of alternating α (1 → 3) and α (1 → 4) glycosidic bonds is also possible [59]. Fucoidan is under development for many biological and biomedical activities as it plays a crucial role in controlling acute and chronic inflammation via mechanism of enzyme inhibition and complement cascade inhibition [59]. Fucoidan has been found to contain several interesting properties that may prevent the disease of cancer [60]. Therefore, a marine-derived fucoidan has been trialed for to assess its efficacy as antitumor activity and immunological properties [61]. Fucoidan can be used for the treatment of osteoarthritis [60] and has also been used successful for application in healing-impaired wound dressing [62].

15.2.7 Carrageenans

Carrageenan represents a family of linear sulphated polymers extracted from some species of red algae (Rhodophyta—Class Gigartinales), mainly from Chondrus, Eucheuma, Gigartina and Iridaea genera [63]. They are sulphated polysaccharides consisting of a backbone derived from galactose with regular but may be unspecific structures, depending on the source and the methods of extraction/modification. Three main types of carrageenan have been classified according to the number of sulphate groups per disaccharide basic unit after alkaline modification: kappa, iota and lambda [64]. Carrageenans extracted from red algae [65] are widely used in food industry but are also suitable for broad spectrum of biomedical and biotechnological application.

15.2.8 Glycosaminoglycans

Glycosaminoglycans (GAGs), polydisperse natural polysaccharides, typically consist of a repeating disaccharide unit constituted by a hexose and a hexosamine. Sulphated GAG is present in a wide range of marine species-like sponges (Porifera) [66] and several classes of fishes (Actinopterygii and others), particularly in sharks,

skate, codfish, salmon and trout [67, 68]. The application of GAGs is increasing in different sectors like biomedical research, biochemical industries, biopharmaceutical and nutraceutical science. Due to its ideal property like anti-pathogenic, anti-tumor, anti-coagulant etc., marine-derived GAGs are being widely studied for application in different biomedical applications.

15.2.9 Chondroitin Sulphate

Chondroitin sulphate is available from a variety of natural sources including terrestrial species (bovine, porcine and chicken cartilage) and marine species [69, 70]. Among marine sources, it has been derived from whales [71], sharks [72], skates [73], squids [74], salmons [75], king crabs [76] and sea cucumbers [77]. Marine invertebrates such as Cnidaria, Polychaeta and mollusks are also a rich source of Chondroitin sulphate [78]. Among them, shark cartilage has been the most common non-mammalian commercial source of chondroitin sulphate. However, scarcity of the raw material and environmental impact are the main hurdle for new developments in the upcoming years [68]. Chondroitin sulphate has become widely used for treatment of osteoarthritis and other regenerative medicine application.

15.2.10 Hyaluronic Acid

Hyaluronic acid, also referred to as hyaluronan is a non-sulfated glycosaminoglycan and is widely found in the intercellular matrix of most connective tissues such as cartilage, vitreous of the human eye, umbilical cord and synovial fluid. Hyaluronic acid is also found naturally in marine ecosystem, like cartilaginous fishes and in vitreous humor of different fish species. Hyaluronic acid can also be produced in large amount from some strains of bacteria such as *Streptococci* through microbial fermentation [79]. The structure of hyaluronic acid consists of alternating disaccharide units of α-1,4-D-glucuronic acid and β-1,3-N-acetyl-D-glucosamine, linked by β $(1 \rightarrow 3)$ bonds [80]. Attempts have been made on hyaluronic acid for various cosmetic and medical uses.

15.3 Marine Biomaterials in Bone Tissue Engineering

Calcium phosphate composite such as hydroxyapatite (HAp) has all the necessary properties for bone tissue engineering such as porosity, architecture, pore interconnectivity etc. with increased efficiency in terms of resorption and made them an ideal material for orthopedics and dental applications [36, 81]. Back in 1974, Roy developed bioceramics (coralline) using a simple biomimetic approach which directly

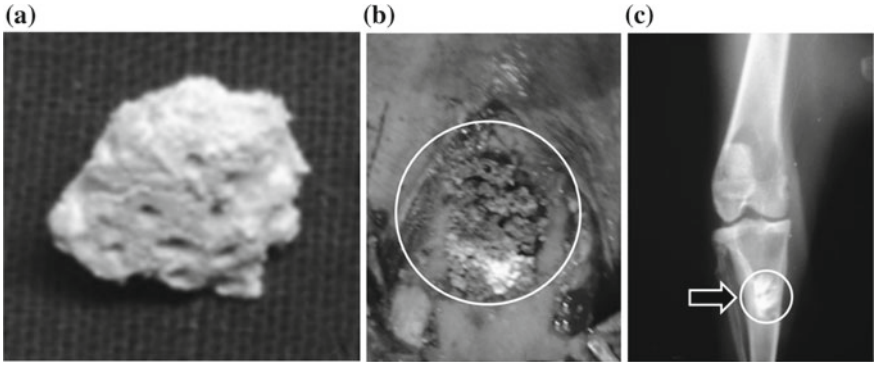

Fig. 15.1 Photograph showing converted marine coral (**a**), porous coral scaffold implanted in proximal tibia bone of rabbit (**b**) and radiograph of radiodense coral scaffold at tibia bone (**c**)

converts coral calcium carbonate skeletons into hydroxyapatite. The derived porous hydroxyapatite microstructure displayed the advantage of circulation of body fluids and the ability of firm attachment with host tissue layer [82]. Figure 15.1a–c shows an example how converted marine coral (Fig. 15.1a), after implantation in proximal tibia bone of rabbit (Fig. 15.1b) positioned and expressed as radiodense coral scaffold at tibia bone from radiograph (Fig. 15.1c).

In addition, hydroxyapatite can also be produced from other marine sources like fish bone and shell [83, 84]. Some of the commercially available HAp based products such as Pro-Osteon™ 500R, Pro-Osteon™ 200R and Interpore 200 are widely used in several biomedical applications [85]. The benefit of using HAp developed from marine origin is that they are predominantly safe, biocompatible, and osteoconductive. So, they can be effectively used as bone graft alternate for bone defect regeneration [39]. Figure 15.2a–c shows an example how converted marine sponge (Fig. 15.2a), after implantation in distal femur bone of rabbit (Fig. 15.2b) positioned and expressed as radiodense converted marine sponge scaffold at the same location established from radiograph (Fig. 15.2c).

Alginate gel is another biomaterial used for bone and cartilage regeneration as they can be introduced with minimal invasion into the body, their capability to fill irregularly defect shapes and sizes, easy to modify with adhesion ligands (e.g., RGD), and sustained release of various growth factors (e.g., BMP, TGF-β) [86, 87]. Sodium alginate/gelatine has been used as direct scaffolding material for bone healing [88] and alginate hydrogels has also been widely used for the transplantation of stem cells in bone tissue engineering [89]. The porous alginate scaffolds can help for vascularization, oxygenation, cell migration, adhesion and proliferation, a fundamental step for bone tissue regeneration [90]. However, alginate in combination with hydroxyapatite is a better scaffolding material [91, 92], as alginate scaffold alone lacks sufficient mechanical strength. In addition, chitosan is a biomaterial which can be reconstructed into several structures like film, fibers, beads and scaffolds [93, 94] and can be combined with different class of materials such as alginate, HAp, cal-

cium phosphate, poly(methyl methacrylate), poly(L-lactic acid), and growth factors for possible application in bone regeneration. Chitosan has been widely studied in bone tissue engineering for its ability to support bone cell growth and deposition of mineral-rich by osteoblasts cells (Fig. 15.3a–c) [95].

Among various marine biomaterials, fucoidan also have several biological applications. It was found in the recent studies that osteoarthritis symptoms were significantly reduced by 52% following oral administration of fucoidan-rich seaweed extract [60]. One of a commercially available fucoidan bone substitute (Lubboc®) is a low molecular weight fucoidan (LMWF), developed to enhance bone regeneration and was reported to be beneficial in supporting human osteoblastic behaviors, collagen type I expression and favors alkaline phosphatase (ALP) activity, thereby improv-

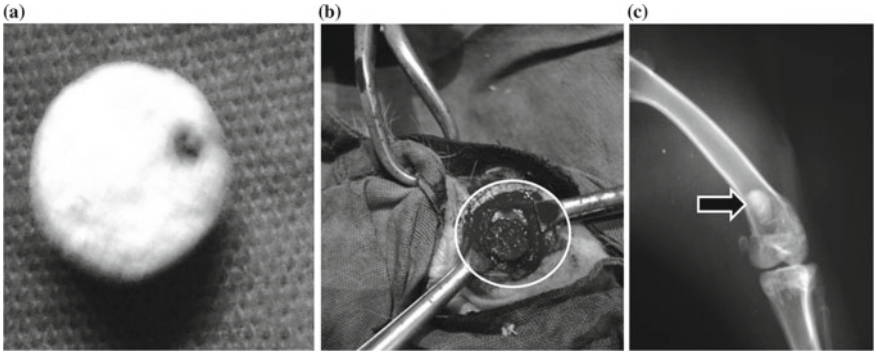

Fig. 15.2 Photograph showing converted marine sponge (**a**), porous sponge scaffold implanted at distal femur bone of rabbit (**b**) and radiograph of radiodense sponge scaffold at distal femur bone (**c**)

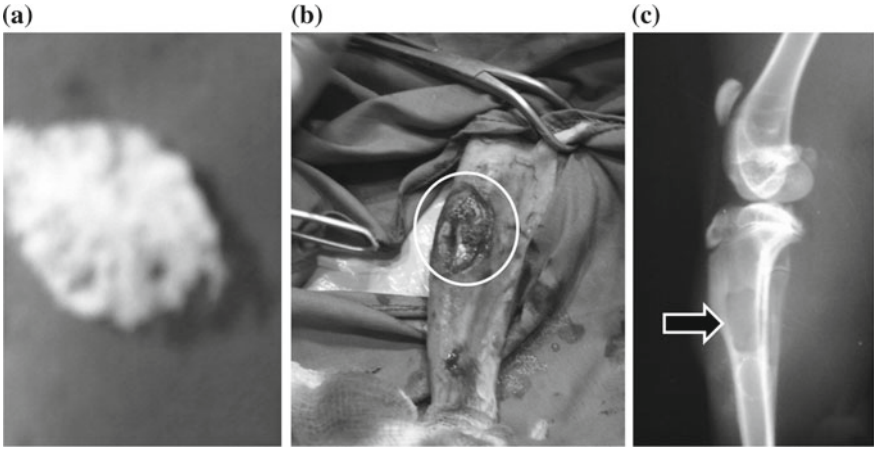

Fig. 15.3 Photograph showing chitosan (**a**), implanted in proximal tibia bone of rabbit (**b**) and radiograph of chitosan implanted proximal tibia bone (**c**)

ing bone mineralization [96]. Biocomposite material made of polycaprolactone-fucoidan showed exceptional cellular proliferation and bone mineralization ability with a controlled-release fucoidan enabling as active biological stimulator for bone regeneration [97, 98]. In addition, it was believed that biosilica from marine origin can provide a suitable material in bone tissue engineering, since it exhibits beneficial anabolic effects on bone forming cells (osteoblasts); but inhibit the function bone-resorbing cells (osteoclasts) [99]. Particularly, biosilica may be advised for an approach which could promote anabolic mechanisms in bone. Further, biosilica activated the expression of important cytokine like BMP2, resulting differentiation of bone progenitor's cell to functional mature osteoblasts [100] and had an adverse effect on osteoclasts, advocating its convenience for treatment of the disease of bone diseases [101].

Several methods and combinations have been used to fabricate marine biomaterials for bone tissue engineering. Biomaterial produced by incorporating HAp with chitin was found to be non-cytotoxic and biodegradable both in vitro and in vivo; thus, proved to be a promising candidate for bone substitute application [102]. Similarly, an experiment with porous chitosan-HAp materials synthesized by a method in an aqueous medium was reported to have good osteoconductivity in vivo and act as promising materials for bone regeneration when used in tibial bone defects in rat [103]. Porous HAp ceramic has also been successfully used for repairing tibial gaps in sheep model as a carrier for autologous bone marrow osteoprogenitor cells [104]. In addition, chitosan/alginate hybrid scaffolds had been successfully used for bone tissue engineering as it improved the structural stability and mechanical strength when implanted in the bone defect and reported to enhance osteogenesis and vascularization [105]. Collagen from marine gastropods, especially the one extracted from different body parts of *Ficus variegate* has been investigated as well, to fabricate a porous scaffold upon crosslinking with various commercial agents, like chitosan, HAp and glutaraldehyde. The resultant composite scaffold can be additionally used in bone tissue engineering and diverse biomedical applications [106]. Chitosan–alginate and chitosan–alginate-fucoidan polymer scaffolds developed by a freeze-drying method were both suitable for bone graft substitute. However, chitosan–alginate-fucoidan provides a better material for bone tissue engineering [107].

15.4 Marine Biomaterials in Dentistry or Dental Application

Some of the common marine products that have potential for dental tissue engineering application are marine sponge skeletons, nacre seashell, Diatom frustules, *Foraminifera* shells, and coral skeletons [12, 108–110]. A massive range of materials and substrates have been studied, like marine collagens and alginates from seaweed; chitosans, chitins, and chito-oligosaccharides from crustacean shells. These marine biomaterials have been extensively employed for several dental applications [31,

48, 111–115]. Nonetheless, there are some less familiar and hidden marine-based materials and substrates that are yet to be explored for implementation in dental hard tissue restitution, cleaning and augmentation roles.

In particular, chitosan has emerged as one of the principle candidates for biomedical applications owing to its significant bioactivity [116], antimicrobial [117], biocompatibility [118] and compatibility to blend with other materials to make a complex in several forms [119]. These properties make chitosan more versatile and serve as an important material for various biomedical field, particularly in dental applications [120, 121]. Gels and hydrogels made up of chitosan can be applied for the treatment of chronic periodontitis and canker sores. Toothpastes, mouthwashes, chewing gums and some other pharmaceutical products based on chitosan and herbs possessed an antimicrobial property on oral biofilm and reduced the concentration of *S. mutans* in the oral cavity [122, 123]. Chitosan-fluoride micro particles augmented absorption of fluoride and further prevents dental cavities. Moreover, chitosan based endodontic cements reduce inflammation and promote dental bone restoration [120].

A number of researches have been conducted to study the effect of utilizing chitosan as a coating material on dental implants. It is believed that chitosan coating may stimulate bone interface interaction by altering biological, mechanical and morphological surface properties [124]. In addition, chitosan coatings may be used as a carrier for local delivery of antibiotics around the implant area. Still, additional works need to be done to authenticate the role of coatings plays in controlling infection vis-a-vis osseointegration [125]. A commercially available chitosan-based dentifrice (Chitodent® (B&F)), which is non-fluoride formulation is reported to significantly reduce tissue loss [126] and NaF- and Sn/chitosan-based dentifrices also prevents the erosion of the dentin organic matrix [127] and enamel. Similarly, the addition of chitosan enhanced the efficacy of Sn^{2+}-based dentifrices for protecting tissue loss in acidic oral environments by providing both anti-erosive and anti-abrasive effects [128]. Others employed chitosan-based hydrogel as a medium for delivery of amelogenin, aiming at rejuvenating the aligned crystal structure for human enamel regrowth [129]. As mentioned earlier, chitosan is also compatible to be blended with other marine derived materials like HAp, alginate, collagen etc. enhancing its biological properties. Some report has demonstrated that nano HAp/chitosan composite scaffold had better cytocompatibility than a pure chitosan scaffold [130] and a chitosan/alginate composite also significantly enhanced mechanical strength of pure chitosan [105]. Eventually, a collaborative research approach should be developed involving tissue engineering, biotechnology, biomolecules and materials science to investigate the further potential of chitosan and other marine biomaterials for dental tissue regeneration applications.

15.4.1 Oral and Maxillofacial Surgery

Recently, biomaterials from marine organisms are being considered highly striking and gaining popularity for craniofacial engineering. Marine collagen from numer-

ous marine sources can be successfully utilized for this field. Collagen comprises the major constituent of the extracellular matrices in all animals and metazoans. Besides its attractive mechanical properties like elasticity, marine collagen exhibited good bioabsorptive property with pore interconnectivity, enabling adherence and proliferation of human Mesenchymal Stem Cells (hMSCs) and resulting in better osteogenic differentiation [131]. Amid marine species, fish produces abundant collagen which can be used in various biological processes. In addition, other marine organisms like coral or sponge contained mineralized porous structures capable of replacing the human bone features. So, collagen obtained from marine sponges can be investigated as a bone substitutes for different bone regenerative approaches (Fig. 15.1) [132, 133].

Evaluation based on biomedical relevance of chitosan and collagen extracted from marine sources for the effectiveness for regenerative medicine demonstrated the biocompatibility biodegradability properties of fish atelocollagen; thus, this proves to be a suitable material in regenerative medicine [134]. In addition, a biomaterial obtained from natural corals called as Biocoral have been successfully applied as an alternative bone graft substitute in maxillofacial surgery [135]. In fact, coral grafts have been used effectively in maxillofacial surgery since 1992 and was reported that the derived marine scaffolds were well accepted vis-a-vis ossified as the calcified skeleton is resorbed when implanted in a number of clinical cases [136].

Successful clinical outcome of an implant derived from marine sources for grafting maxillary sinus depends on the formation of new essential autogenous bone and bone mineral density. Naturally occurring HAp graft material of marine algae with autogenous bone has been used in the sinus augmentation procedure to support dental implants under occlusal loads. Histologic and histomorphometric data supported the discovery of new bone formation with adequate bone mineral density at the area of grafted maxillary sinus [137]. In another report, a certain variety of coral, Porites of the Madreporaria reef builders in Hainan Province, was investigated as a bone graft substitute for maxillofacial surgery. The clinical results demonstrated that the coral is well tolerated by the body and no sign of adverse host tissue response was reported. In addition, the coral played a crucial role as a bone conductor and successfully regenerated the bone defects after surgical implantation; which proves to be a useful bone substitute [138]. HE800 exopolysaccharide (HE800 EPS) is also another unique marine biomaterial, a glycosaminoglycan secreted by a deep-sea hydrothermal bacterium exhibiting promising features by resembling hyaluronan. It was observed that this HE800 EPS family could be employed as a novel biotechnological source of glycosaminoglycan-like compounds suitable for fabricating biomatrices and in the development of drugs for tissue engineering applications [139].

15.5 Marine Biomaterials in Cartilage Tissue Engineering

Marine origin biomaterials including those are derived from *Millepora dichotoma*, the net fire coral and the coral skeleton of *Porites lutea* provides novel scaffolding

materials for cartilage tissue engineering [140]; and in particular the coral skeleton of *Porites lutea*, has been extensively investigated in the development of scaffolds for bone tissue engineering [141]. The skeleton of *Millepora dichotoma* has also been used effectively as suitable implant in chondral and subchondral remodeling [142]. It is believed that the coralline lattice possesses all the ideal characteristics required for bone and cartilage graft substitute including biocompatibility, biodegradability and temporary mechanical strength. Furthermore, it has the property to stimulate cellular invasion, adherence, proliferation [141, 143] and an osteogenic capacity [143].

Among the commonly available marine biomaterials, chitosan has been extensively used in tissue engineering application due to its biocompatibility and biodegradability [144, 145]. Additionally, the structure of chitosan resembles various GAGs (glycosaminoglycans) found in articular cartilage [146]. Marine polysaccharides (CHT/HAp scaffolds) can be successfully used in cartilage tissue regeneration. Blending these two materials provide a stable conjugate by an ionic interaction between the positively charged chitosan and the negatively charged hyaluronic acid to obtain a biomimetic matrix for chondrocytes [147–149]. It was further concluded that chitosan-based hyaluronic acid polymer fibers developed by the wet spinning method significantly improved chondrocyte adhesion, proliferation, and the synthesis of aggrecan and type II collagen, compared to chitosan fibers alone [148]. Moreover, chitosan not only can provide appropriate microenvironment for cartilage tissue regeneration but can also stimulate cellular attachment and proliferation, promoting tissue regeneration through varieties of ways [147]. Chitosan modified with poly l-lactide-co--caprolactone (PLCL) also improved cell biocompatibility and cartilage tissue regeneration with better quality [150], besides significantly enhancing the excretion of aggrecan and type-II collagen [151]. Chitosan can also be blended with other materials to form natural matrix such as gelatin and silk fibroin. It was demonstrated that silk fibroin/chitosan scaffold can provide excellent carriers for stem cells to repair cartilage defects [152], and the chitosan-gelatin (1:1) complex scaffolds cross-linked by water-soluble carbodiimide may also enhance cartilage regeneration [153].

Carrageenan is another marine biomaterial that received considerable attention in cartilage tissue engineering. This marine polysaccharide specifically has several advantages for cartilage regeneration owing to its structural resemblance to glycosaminoglycans (GAGs), one of the main constituents of the ECM of the cartilage tissue [154, 155]. It was intimated that incorporation TGF-β1 in the carrageenan-based hydrogels can stimulate cell–cell interactions, chondrocyte proliferation and differentiation of cells [156, 157], as well as production of proteoglycans and other components of cartilage matrix [158]. Furthermore, the carrageenan-based hydrogels incorporating TGF-β1 enhanced the cell viability and proliferation, and increased the expression level of chondrogenic differentiation markers, which promote the competency of κ-carrageenan for cartilage tissue engineering.

15.6 Marine Biomaterials in Wound Regeneration

Compounds obtained from aquatic species possess promising potential for wound tissue engineering. Accordingly, materials derived from marine organism began to be used in soft tissue regeneration [159, 160]. Among them, collagen is one biomaterial which plays a vital role when used as scaffolds for different types of cells by promoting cellular attachment, migration, proliferation, differentiation, survival [161]. Since the collagen used in tissue engineering applications today is still obtained from terrestrial animal tissue like bovine and porcine skins and tendons, the collagen derived from these sources may cause possible disease transmission [48]. Consequently, collagen derived from marine source provides an advantage and received considerable attention for the development of tissue engineering substitute due to its lack of disease transmission to human. Recently, experiments based on marine-derived collagen scaffolds for skin tissue regeneration have been carried out extensively and reported high potential of collagen in clinical applications [162]. Collagen sponge derived from salmon fish was studied for tissue engineering applications as an artificial dermis scaffold. Based on their study, a positive result was reported; and due its promising properties for tissue regeneration and safety as a biomaterial, much interest is being paid to explore the potential, suitability and acceptability of marine collagen in the regeneration of skin tissues [163].

Alginate is another marine biomaterial that can be used for wound healing application as alginate has been found to possess favorable properties for skeletal muscle regeneration [62, 164, 165]. Commercially, alginates are extracted from several species of brown algae such as *Laminaria hyperborea, Ascophyllum nodosum, Macrocystis pyrifera, Laminaria japonica, Eclonia maxima, Lesonianegrescens*, and *Sargassum* [166]. Alginate has an attribute to retain a moist environment due to its hydrophilic property by adsorption and desorption process and subsequently alginate hydrogel can be employed effectively for dressing in wound management [167]. It has also been regarded an encouraging agent in soft tissue applications as a substitute or alternate grafting, especially in deep-thickness wounds.

Similar to alginate, chitosan has been widely used for topical wound dressing due to its desirable qualities like non-toxicity, biodegradability, biocompatibility, and antimicrobial property, which enhances the wound healing process [168]. Chitosan and its derivatives are known to have excellent antimicrobial activities against most of living microorganisms like yeast, fungi and bacteria [169, 170]. The promising biological properties of this biopolymer in wound healing, hemostasis, and immune enhancement for application in wound healing and skin tissue engineering applications draws the attention of many scientists. For example, chitin obtained from crab shells has been blended with other marine-derived compounds to develop a hydrogel for wound dressing. It was reported that the hydrogel dressing provides good moist healing environment, resulting a significant increases in granulation tissue and vascularization in full-thickness wounds created in rats [62]. In addition, a nanofibrous scaffold made of chitosan obtained from crab shell blended with PLGA and

polyvinyl alcohol (PLGA-chitosan-PVA) has shown to be an ideal agent for skin tissue engineering substitute [171].

Hyaluronic acid can also be obtained from marine organisms for various biomedical applications owing to its outstanding contribution to biological functions such as cell adhesion, migration, differentiation, and proliferation in tissue development [172]. It is known that hyaluronic acid has a provision to facilitate the diffusion of nutritional supplies in the early stages of wound healing and eliminate metabolic waste products from the tissue [173]. In the past decades, extensive studies have reported on numerous numbers of prospective for skin tissue engineering. As a result, different varieties of tissue-engineered skin substitutes such as hydrogels, sponges, and meshes with outstanding biological properties have been developed [174, 175]. Further, fucoidans, a sulfated polysaccharides [176], is known to have excellent characteristics for wound healing and fabrication of skin tissue-engineered substitutes. Marine derived chitosan composite film containing fucoidan obtained from Fucus vesiculosus has been developed for dressing of dermal wound. Wound dressing with the composite film produced perfect mechanical and biological properties such as excellent dermal capillary formation, re-epithelialization, wound regeneration and rapid wound closure in superficial dermal burn wounds [177]. Currently, carrageenans are also widely utilized in many pharmaceutical industries since they have been approved by the Food and Drug Administration (FDA) as a safe biological material [178, 179]. Carrageenans have been considered as an ideal material for wound healing acceleration and could also be actively utilized for drug delivery into a wound. Further, it is highly rated as good material for wound dressing owing to its excellent antimicrobial activity and moisture absorption [180, 181].

15.7 Marine Biomaterials for Local Drug Delivery Applications

Local drug delivery is the process of delivering the pharmaceutical agent into the specific side or place to avoid other side effects. Marine-derived polymers are biocompatible, have desirable surface architecture and mechanical strength which are the essential features for a successful scaffold to be used as a carrier for drug delivery [44, 182, 183].

Chitosan and alginate have been probably the two most commonly employed marine-derived polymers for construction of drug delivery particles [184–186]. Chitosan is an excellent biopolymer as excipient for oral drug formulation and vehicles for parenteral drug administration. In addition, chitosan is also used to produce sustained release drug delivery systems via other routes like nasal, ophthalmic, transdermal, and implantable devices [187]. It was reported that chitosan and its derivatives have a great potential as a biodegradable system for delivery of hydrophobic drugs like ketoprofen in a pH-sensitive controlled release [188]. Some other studies have also demonstrated that chitosan has the ability to enhance and prolong the absorption

of hydrophilic drugs when administered orally [189] and through pulmonary routes [190]. Chitosan combined with PEG (polyethylene glycol) can boost the biocompatibility of chitosan, especially by reducing chitosan toxicity, enhancing protein adsorption, as well as adhesion, growth and proliferation of cell [191]. Other studies showed that chitosan and copolymers also have the ability to deliver certain drugs such as insulin [192, 193]. It is possible to construct drug delivery systems for the intracellular release of the genetic material by using chitosan and its chemical derivatives (like PEI-PEG (polyethylenimine and polyethylene glycol)-chitosan-copolymer) for the intracellular release of the genetic material, which open a new way of the treating various genetic diseases for in vivo gene delivery [194].

Similarly, alginate has been used as an excipient for production of tablets in many pharmaceutical industries to enhance protection and stabilization of the drug. Sodium alginate is particularly used in the pharmaceutical industries to manufacture tablets, especially when the drug is not soluble in water. In addition, sodium alginate may also be used for the purpose of extending and prolonging the release of drug [33]. Based on various studies, it has been demonstrated that the bioavailability of drugs encapsulated in alginate hydrogels is higher as compared to direct application of the free drug at the lesion site, thus enhancing the efficacy of healing [195]. Alginate construct as microparticles also has the ability to incorporate with different bioactive agents, especially proteins, as alginate exhibit an excellent potential for the delivery of protein drugs. Besides, alginate microspheres have the capability to encapsulate or load the desired amount of protein, transporting it to the targeted sites, and controlling the kinetics of the protein release [196]. In addition, alginate microparticles possess the ability of retaining large amounts of drug and protecting it from any proteolytic attack [197, 198]. Alginate complexes also showed a promising result to construct drug delivery systems (especially nanoparticles) for genetic material in gene therapy treatments [199].

The use of carrageenans as a biomaterial has also been considered for diverse medical applications in drug/growth factor delivery systems [200], immobilization of enzymes [201], and loading of several cell types for in vivo delivery [202]. Carrageenan is a marine derived material commonly used in pharmaceutical industry for the construction of drug delivery systems and cell capsules for cell therapies [203]. For instance, Sezer and Akbuğa developed a new microspheres delivery system by cross-linking of fucoidan with chitosan (Fucosphere) and reported that the extent of drug release is dependent on the concentrations of the polymers and protein [204]. Similarly, novel chitosan/fucoidan nanoparticles with antioxidant properties for antibiotics delivery had also been developed [205]. An attempt was made to synthesize chitosan/fucoidan microcomplex hydrogel for the delivery of heparin binding growth factors by seizing the benefits of the interactions between chitosan and fucoidan. The growth factors have high affinity towards this complex hydrogel as well as sustained release profile characteristics [206]. On the other hand, it was also reported that the release of growth factors from the chitosan/fucoidan hydrogel promoted the neovascularization in vivo. Consequently, it has been suggested that the chitosan/fucoidan pH-sensitive nanoparticles can be used for oral administration

of drugs. Furthermore, the fucoidan-chitosan complex nanoparticles have increased potential for the delivery of anticoagulant agents [207].

Among the biopolymers, collagen extracted from marine origin can also be used as drug delivery systems. In addition of being a promising biodegradable polymer, it is also a natural biomaterial with haemostatic and wound healing properties [208]. A number of drugs such as antibiotics and antiseptic (tetracycline, doxycycline, rolitetracycline, minocycline, metronidazole, ceftazidine, cefotaxime, gentamicin, amikacin, tobramycin, vancomycin, and chlorhexidine), statines (rosuvastatin), vitamins (riboflavine), parasympathomimetic alkaloid (pilocarpine) can be incorporated in the collagen biomaterials [209–211]. The most documented collagen-based drug delivery systems are the hydrogels and matrices. Hyaluronic acid, also referred to as hyaluronan is another biomaterial with a promising behavior that can be used in the production of drug delivery systems. Injectable hydrogel composed of hyaluronan-tyramine conjugate was developed to encapsulate drugs with high degree of biodegradability [212]. Similarly, a new hyaluronidase-incorporated hyaluronic-tyramine hydrogel was developed as a delivery vehicle for trastuzumab, an antibody drug against breast cancer [213]. Similar to other polyanions, hyaluronan can be complexed with polycations such as chitosan to form nanoparticles [214] and microspheres [215]. Recently, hyaluronic acid-chitosan nanoparticle has also been examined as a new approach for the treatment of ocular disorders. This novel nanoparticle has been developed by means of electrostatic interactions for ocular gene therapy to treat the cornea and conjunctiva [216]. From a biomedical perspective, the results revealed the possibility of utilizing nanoparticle as gene delivery device in the treatment of various types of ocular diseases in human [217].

Chondroitin sulfate is another outstanding marine biomaterial that has been suggested and developed for a variety of biomedical applications. Hydroxyapatite-chondroitin sulfate hybrid mesoporous material was synthesized for controlled and sustained release of antitumor drugs. The synthesized hydrogels were reported to be biocompatible as there was no inflammatory response observed during implantation. It is also biodegradable through enzymatic activity. Based on a recent study, it was demonstrated that porous tubular structures could be used effectively for the delivery of chemotherapeutics despite the fact that 3D implant and spherical design are commonly used for drug delivery [218]. High encapsulation capacity of up to 91% efficacy was reported during the sustain release of doxorubicin hydrochloride and proved the potential application of this material as controlled drug delivery system for chemotherapy treatments.

15.8 Conclusion

Oceans and marine bodies are abundant source of biomaterials and possess immense potential for tissue engineering application. However, since the field of marine biotechnology is still developing, its full potential has not been yet achieved. So, further research and future prospect may pave a way for major break-through to

understand the resources better in order to solve various health related issues affecting the human race.

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Chapter 16
Composites Containing Marine Biomaterials for Bone Tissue Repair

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Abstract In recent years, a striking development has been achieved in marine biomaterials for bone tissue repair. Marine sources have proven to be non-polluting and versatile for biomedical applications. Bone tissue engineering is a promising alternative for treating bone ailments caused due to trauma and surgical intrusions. Biocomposites comprise of biodegradable and biocompatible materials and mimic the architecture of bone and support regeneration. Significant sources of marine biomaterials are fish, invertebrates, fungi, corals, etc. Bone defects are treated using marine biocomposite polymers such as chitosan, collagen, alginate, gelatin, and ceramics. Chitosan is anti-microbial and bioactive; hydroxyapatite and collagen are significant constituents of bone, and alginate boosts mechanical strength and structural integrity of biocomposites. This chapter accounts for the source and types of biomaterials from marine fauna, the fabrication of biomaterials as scaffolds and their biological activity in enhancing bone repair in vitro and in vivo.

Keywords Alginate · Alizarin red staining · Alkaline phosphatase staining · Biocomposites · Bone defects · Bone tissue engineering · Chitosan · Gelatin · Hydroxyapatite (HAp) · Marine sources · Von Kossa staining

16.1 Introduction

Bone is a mineralized, rigid connective tissue that makes up the structural framework of the human body. It has an in-built regeneration and remodelling capacity throughout its lifetime. This special ability of bone makes it highly dynamic towards establishing homeostasis [1]. Other than that, bone also has several secondary functions such as mineral storage, blood pH, and calcium regulation. Bone constitutes of a mineral phase known as hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$), organic phase mainly composed of type I collagen and other inorganic components such as water [2].

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Clinically, bone fracture healing is considered as bone regeneration process where a number of cells are involved in extracellular and intracellular signalling pathways and work towards bone repair. Critical size defects as a result of external trauma, abnormal medical conditions, and diseases are areas of interest in the application for assisted bone tissue repair. Severe traumatic injuries result in significant tissue loss, which needs highly complex medical reconstructive approach [3]. It was estimated that 200 million people are affected by osteoporosis, and 8.9 million suffer from bone fractures annually worldwide [4]. This instigates the necessity of effective medical intervention for bone tissue repair.

Bone remodelling is generally guided by three types of cells—osteoblasts, osteocytes and osteoclasts. The homeostasis and dynamic nature of bone tissue are balanced by the activities of osteoblasts and osteoclasts. Osteoblasts are of mesenchymal stem cell (MSC) origin, and they share their lineage with other cells such as adipocytes, myoblasts, and chondrocytes [5, 6]. They are specialized cells that differentiate into bone cells and make up the continuous bone cell layer. Osteoblasts primarily form bone cell layer by synthesizing extracellular matrix (ECM) proteins such as collagen I. When osteoblasts are trapped inside the ECM that they have secreted, they essentially become osteocytes. Osteocytes have an important role in regulating the dynamic nature of bone and mineral homeostasis [7]. In homeostasis, old bone tissues are first removed by osteoclasts before they are replaced by new bone cells. Osteoclasts resorb the mineral bone tissue and tend to physiological remodelling. An over-expression of osteoclast cells leads to brittle bone disorders like rheumatoid arthritis [8, 9].

Osteogenesis or ossification is the process in which new bone tissue is layered upon by osteoblasts [10]. This is the primary process in bone remodelling, and it can be classified into intramembranous ossification and endochondral ossification [11–14]. Intramembranous ossification is the process where the bone is formed directly over the mesenchymal tissue. It is involved in the fracture healing of skull, jaw, and clavicles. Endochondral ossification needs a cartilage tissue as a precursor template as it is invaded by the blood vessel, osteoblasts and osteoclasts to form a new bone. It is involved in the lengthening of bones and plays a major role in the bone healing process.

Classically, bone repair has been supported by tissue transplantation or grafting techniques. Bone grafting is an invasive procedure that involves replacing a missing part of the bone tissue with the host's tissue (autograft) or from an individual other than the host (allograft). Autograft is considered the best option since it involves self-donated tissues that are highly biocompatible. Grafting techniques pose serious medical conditions like immunogenic reactions leading to graft rejection, pain, and plate extrusion. Hence, bone grafting techniques have low success rates making them inept. An alternative method for getting bone tissue is explored by bone tissue engineering [15–21].

Bone tissue engineering involves the utilization of biomaterials such as polymers, ceramics, osteogenic stimulants, stem/bone cells and other cell signalling components aid in bone regeneration. The polymeric or bioceramic based scaffolds are widely used for providing a suitable milieu for bone regeneration.

A biopolymer should be osteoinductive, osteoconductive and osseointegrative [22–26]. Osteoinduction is the process through which osteoprogenitor cells differentiate into osteoblasts and starts forming new bone tissue. Osteoconduction is the process through which osteoblastic cells infiltrate the scaffold and form new bone structure. Osseointegration is the process in which a direct interface is formed between the boundary of the bone and the implant [27]. Both natural and synthetic polymeric complexes are used, with varying properties such as biocompatibility, surface morphology, swelling ratio, protein adsorption and degradation rate. Natural polymers include chitosan, gelatin, alginate, collagen and cellulose, and synthetic polymers include polycaprolactone (PCL), polyvinylpyrrolidone (PVP), and polylactic acid (PLA) [17, 20, 28, 29]. Although both natural and synthetic polymers are used in bone tissue engineering, natural polymers are regarded to have better biocompatibility and lesser toxicity when compared to synthetic compounds. There is a wide range of natural polymers that have been used in bone tissue engineering applications and sometimes the polymers in itself are regarded to be osteogenesis inducers [30]. More recently, the phytocompounds like flavonoids have been reported to possess some osteogenic properties upon incorporation in scaffolds for bone tissue engineering applications [31–33].

About 70% of the earth is covered by ocean, making half the global biodiversity to be marine. Hence, marine biodiversity is an excellent source for many novel compounds [34]. Evolution of many algae, fish, sponge, and crustaceans have enabled them to acquire defence systems against prevailing predators based on the use of a variety of biomolecules that enable them to survive hostile environment which includes stressful conditions such as high salinity, temperature, pressure and light. Many macromolecules like proteins, glycoproteins, and polysaccharides have also been identified to have biotechnological applications that were intended to be cell surface markers, differentiation and developmental proteins in the marine organisms [35]. For example, agar from marine sources has extensive pharmaceutical applications such as drug delivery systems, bone tissue scaffolds and as suspending agents [36].

16.2 Chitosan

Chitosan is a typical semi-crystalline polysaccharide that is abundant in marine biodiversity. It is a randomly linked linear polysaccharide that consists of β-(1→4)-D-Glucosamine and N-acetyl-D-glucosamine. Chitosan has extensive biomedical applications due to its non-toxicity, film-forming properties, biocompatibility, and physical characteristics. Among numerous polysaccharides, chitosan is produced in large scale about 10^{11} tons per year, making them the highly abundant organic compound on the earth. Chitosan is primarily derived by deacetylating chitin (β-(1→4)-N-acetyl-D-glucosamine). It is the only known pseudo-natural cationic polymer [37]. Crabs, clams, oysters, shrimps and several other crustaceans are essential sources of chitin. Chitin is isolated by treatment with hydrochloric acid to remove calcium car-

bonate followed by alkali treatment to remove proteins on the shells. Upon drying, a powdered form of colorless α-chitin is extracted. Chitin is classified into α- and β-forms based on their polymeric chain orientation. α-chitin is the predominant form of chitin made up of an anti-parallel chain of two N, N'-acetylchitobiose units [38]. Since β-chitin is found in very few organisms such as squid pens (*Ommastrephes bartramii*), α-chitin is widely used for the commercial preparation of chitosan [39]. Chitosan is prepared by de-acetylating chitin with further alkali treatment at high temperature [40].

Chitosan in insoluble in both organic and polar solvents under normal conditions, however, it is soluble in aqueous acids [41]. It has wide applications in pharmaceutical industries due to its physical and biological properties. Because of its cationic nature, chitosan exhibits good mucoadhesive and permeation enhancing properties, thus deeming them to be a fit model for sustained release of drugs in drug delivery systems [42]. Chitosan has been proven to possess anti-microbial properties that make the polymer desirable in wound healing applications. It contributes to the mineralization of bone. Chitosan was observed to promote differentiation of osteoblasts from mesenchymal lineage [43]. Chitosan gel was applied to bone matrix along with collagenous proteins, which showed a statistically significant increase in the mineralization of periodontal regeneration [44, 45].

The architecture of chitosan can be invariably molded into the porous structure by using techniques such as lyophilization, electrospinning, solvent casting, 3D printing, etc. Natural polymers derived from marine sources can be blended with chitosan for enhanced physical and biological characteristics of the scaffolds to improve osteoblast differentiation [46]. This chapter focuses on the studies involved using chitosan and its composites such as hydroxyapatite (HAp), gelatin and alginate scaffolds for bone tissue engineering applications. Table 16.1 provides information about the sources and applications of the above materials.

16.3 Hydroxyapatite, Gelatin and Alginate

HAp ($Ca_{10}(PO_4)_6(OH)_2$) is a prominent ceramic used in biomedical applications. HAp can be extracted from salmon fish bones by alkaline hydrolysis [50]. Fish bones from swordfish (*Xiphias gladius*) and tuna (*Thunnus thynnus*) were frozen and deprived off their organic substances. The dried bones were then calcined in a furnace, by slowly increasing the temperature from 600 to 950 °C. After the calcination temperature reached, the bones were stored isothermally for 12 h followed by cooling them at a rate of 20 °C/min. The calcined samples were then subjected to ball milling for 1 min to yield powdered HAp [51]. HAp has significant resemblance physically and chemically to the inorganic constituents of human bones and teeth. It has excellent bioactivity, biocompatibility, slow biodegradability and good osteoconductive and osteoinductive nature [52]. HAp is the most thermodynamically stable calcium phosphate ceramic compound in solution. Further, in the solution, it has its pH, temperature, and composition closest to that of the physiological fluid.

Table 16.1 Sources and applications of polymers

Polymer	Marine source	Taxonomic name	Applications
Alginate [47]	Brown algae	1. *Laminaria hyperborea* 2. *Laminaria digitata* 3. *Laminaria japonica* 4. *Ascophyllum nodosum* 5. *Macrocystis prrifere*	Wound dressing; tissue engineering
Chitin [41]	Crabs; oysters; shrimps; krills; crawfish	1. *Brachyura* 2. *Ostreidae* 3. *Penaeidae* 4. *Euphausiacea* 5. *Cambarus monongalensis*	Water treatment; agriculture; food preservation; cosmetic industry; veterinary medicine
Hydroxyapatite (HAp) [48]	Coral; fish bones	1. *Coralline officinallis*	Dentistry; tissue engineering
Collagen/Gelatin [49]	Marine sponges; jellyfish	1. *Chondrosia reniformis* 2. *Medusozoa*	Hydrogels for tissue engineering; biodegradable films

Moreover, it is non-toxic, non-inflammatory, and non-immunogenic [53]. Due to the above-mentioned properties, HAp has greater bond formation ability with host tissues when compared with other bone substitutes, such as allograft or metallic implant. It has a variety of biomedical applications such as matrices for controlled release of the drug; ability to form a direct chemical bond with surrounding tissue. Hence, HAp has been widely used to repair hard tissues, coat implants, and acts as fillers in bone, bone repair and bone augmentation [54, 55].

Gelatin is a natural biopolymer partially degraded product of collagen obtained from its hydrolysis. It is derived from fish scales by acid/alkali hydrolysis. The collagen tissue from fish scales was first washed to remove impurities and non-collagenous substances, followed by acid/alkali treatment. Acid treatment yields type A gelatin and alkali treatment yields type B gelatin. Then, gelatin was subjected to water extraction to yield purified gelatin polymers [56]. It still retains some signals such as the Arg-Gly-Asp (RGD) sequence of collagen which may promote cell adhesion, proliferation, and differentiation [32, 57]. Glycine, proline, and hydroxyproline present in gelatin promote adhesion of cells and upon tailoring it to other polymeric scaffolds would have potential benefits in promoting cellular interaction and subsequent tissue growth. Gelatin has also been used in foods, cosmetics, pharmaceuticals and medical fields. The advantages of gelatin over other polymers are (1) commercially available at low price, (2) readily available, (3) biocompatibility, (4) biodegradability, and (5) low antigenicity and no hazardous by-products upon enzymatic degradation. Moreover, gelatin acts as excellent drug delivery vehicles and cross-linkers due to the presence of a greater number of accessible, functional groups for modification [58].

Alginate is an anionic polymer which is used widely in bone tissue engineering. It is a biopolymer substantially found in seaweeds mainly brown algae (*Phaeophyceae*). The seaweeds were subjected to alkali treatment, typically with NaOH. Calcium chloride was mixed with the solution to precipitate out alginate. The precipitated alginate was purified by HCl treatment which yielded alginate. Following this purification process, water-soluble sodium alginate was produced [59, 60]. Alginate scaffolds are primarily used in the form of hydrogels in bone tissue engineering. Alginate is the second widely used polymer after chitosan for biomaterial applications. Alginate is composed of guluronic acid and mannuronic acid. It is biocompatible, water-soluble and biodegradable under normal physiological conditions [46]. Further, alginate is non-toxic and non-immunogenic. It is one of the best-known polymers to treat the loss or failure of organs due to its suitable scaffold-forming property. Additionally, due to its source abundance and low price, it has been widely used in the food industry as emulsifying agent thickener and as a tissue engineering material especially for orthopedic applications [61]. Alginate can be easily fabricated into any form such as hydrogels, microspheres, microcapsules, sponges, foams and fibers which are useful for tissue engineering and drug delivery applications [32, 62].

16.4 Fabrication Methods

Marine biomaterials have a high scope of scaffold formation in tissue engineering. These scaffolds can act as templates which aid in cell adhesion and growth. They also act as a mesh which promotes migration, transport of cells and binding of bioactive molecules for tissue regeneration [63].

There are many methods to fabricate scaffolds based on its intended applications such as solvent casting, lyophilization, phase separation and fiber bonding to create porous scaffolds [64]. Solvent casting involves a polymer/salt or organic solvent mixture after which the solvent evaporates to form a scaffold. It has a simple and inexpensive method of preparation [65].

There are two methods to prepare scaffolds using solvent casting. In one method, the mold is dipped and drawn out of a polymeric solution after a period whereas in the other method the solution is poured into a mold and after a particular time solvent evaporates to form a polymeric membrane layer which adheres to the mold [66]. Fiber bonding is a method to prepare scaffolds resulting in a large surface area, which make them suitable for cell attachment and provide sufficient space for the regeneration of ECM [67]. This method requires two polymers where the primary polymer fibers are bound at their cross points using a secondary polymer. Lyophilization is a commonly used method for the preparation of scaffolds [68]. It involves the dissolution of polymer followed by freezing which results in the formation of ice crystals [65]. When the iced polymer is subjected to sublimation, the original space that is occupied by crystals is emptied, and it leads to the formation of the porous scaffolds [29, 55, 69]. Electrospinning is another method resulting in the formation of polymeric fibres by converting viscoelastic solution using a high electrostatic force [21, 70–72]. In this method, a viscous solution of a polymer is released at a slow rate by a syringe pump to be electrospun. The end-product is continuous nanofibers, and it is used in tissue engineering field as a drug delivery vehicle [27, 33]. Therefore, it could be very advantageous to use marine sources to their full potential to fabricate scaffolds based on recent tissue engineering applications.

16.5 Mechanical Properties

The mechanical property of the bone varies according to its location and type. Young's modulus of cortical and trabecular bone ranges between 15 and 20 GPa and between 0.1 and 2.0 GPa, whereas the compressive strength of cortical and trabecular bone ranges between 100–200 MPa and 2–20 MPa, respectively [73]. Hence to fabricate scaffolds for bone healing and regeneration, its mechanical properties should be taken into account. Scaffolds must have sufficient mechanical strength to support bone regeneration, repair, and maintenance of the bone's integrity at the site of implantation in vitro and in vivo. Construction of porous scaffolds should have an adequate balance between material porosity and mechanical strength. In the

following sections, we discuss the biocomposites along with their mechanical properties used in bone tissue engineering applications.

16.5.1 Chitosan/Hydroxyapatite

Studies have shown that when scaffolds of chitosan are composited with HAp, it improved its mechanical properties and biological response of osteoblasts [74]. The chitosan/HAp composite scaffolds had an excellent mechanical strength with the compression strength of 9.41 ± 1.63 MPa and elastic modulus of 0.17 ± 0.02 GPa. Further, the chitosan/HAp scaffolds showed strong flexure properties with a tensile strength of 3.12 ± 0.12 MPa. These mechanical properties match the compression performance of trabecular bone which gives bone the unique ability to withstand dynamic loading [75]. Chitosan/HAp/magnetite (Fe_3O_4) nanocomposites were subjected to mechanical testing. These scaffolds showed remarkable mechanical properties of compressive strength 23.6 ± 2.1 MPa, compressive modulus 0.6 ± 0.1 MPa and compressive toughness of 4.8 ± 0.3 (MJ/m^3). Hence, by blending chitosan with HAp, it was possible to bring its mechanical strength to the range of human bone [76]. The mechanical properties were tested for chitosan/starch/nano-HAp biocomposites. The chitosan/starch/nano-HAp scaffolds showed an improved compressive strength of 71.84 MPa, compressive modulus of 1326.5 MPa, the tensile strength of 7.36 MPa and tensile modulus reached of 117 MPa [77].

16.5.2 Chitosan/Gelatin

Addition of gelatin to chitosan has shown to improve its mechanical strength. Gelatin being a negatively charged polymer forms polyelectrolyte complex due to ionic interaction between these two molecules and improves the mechanical strength [74]. The mechanical properties of chitosan/gelatin/β-TCP scaffolds were characterized by uniaxial compression test, and these scaffolds had the compressive strength of 0.12 MPa. These properties allowed the chitosan/gelatin/β-TCP scaffolds in the acceleration of bone regeneration and improved the mechanical stability of the target site during the mineralization phase [78]. The nanocomposite scaffolds containing chitosan, gelatin, and $nSiO_2$ showed an increased mechanical property nearly three times more than its individual composite value having a tensile strength of 0.9 MPa [79]. Due to this improved mechanical property, the nanobiocomposite scaffolds served to provide sufficient strength and stiffness that bear in vivo loads during bone regeneration [80]. In another study, silibinin-loaded chitosan nanoparticles incorporated in alginate/gelatin scaffolds showed the high compressive stress of approximately 0.52 MPa. These enhanced mechanical properties of the scaffolds make them suitable for their application at the sites of injury and repair, as the scaffolds structural integrity and stiffness have direct effects on cell regeneration and differentiation [32].

16.5.3 Chitosan/Alginate

Chitosan blended with alginate has shown to increase its structural integrity or mechanical strength by forming a stable bond with alginate. Further, the composite exhibited the swelling properties to support cell attachment and tissue regeneration. Porous chitosan/alginate scaffolds were subjected to stress-strain relations from which the compressive strength and Young's modulus were evaluated. The constructed natural polymer-based complex scaffolds showed significantly improved mechanical strength with a porosity of 92%. They attained a compressive modulus of 8.16 MPa and yielded a strength of 0.46 MPa. Their structural stability was due to the strong ionic bond formation between the amine groups of chitosan and the carboxyl groups of alginate [81]. With the inclusion of $nSiO_2$ in chitosan/alginate scaffolds, the compressive stress and Young's modulus analysis were performed. They exhibited enhanced compressive stress and Young's modulus of 8.99 ± 0.016 MPa and 8.16 ± 0.567 MPa, respectively [82]. Due to their optimal mechanical properties, these scaffolds served as an excellent temporary skeletal frame when inserted into the area of bone loss or bone injury to support and induce bone tissue regeneration [25].

16.6 In Vitro Studies

In vitro studies are not only an important first step toward testing a hypothesis, but these studies also provide valuable insight that allows one to better tailor subsequent in vivo studies. Study outcomes tend to be crucial in the development of surgical instruments, medical devices, novel therapies, and procedures. Also, in vitro studies provide data that is essential for functional validation, FDA applications, and clinical trials. In vitro experiments involved in bone tissue engineering is being carried out at the cellular and molecular levels.

16.6.1 Cellular Studies

Before performing various staining procedures for the determination of osteoblast differentiation and mineralization by scaffolds, it is essential to carry out the biocompatibility/cell viability test such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay. The MTT assay is based on the reduction of tetrazolium by living cells. The cleavage of tetrazolium rings turns the pale yellow MTT into dark blue formazan crystals. The concentration of the crystals formed is directly proportional to the number of active cells. A study was carried out using chitosan/HAp composite scaffolds to measure cell viability. The results showed that the composite scaffolds were cytocompatible and no morphological change

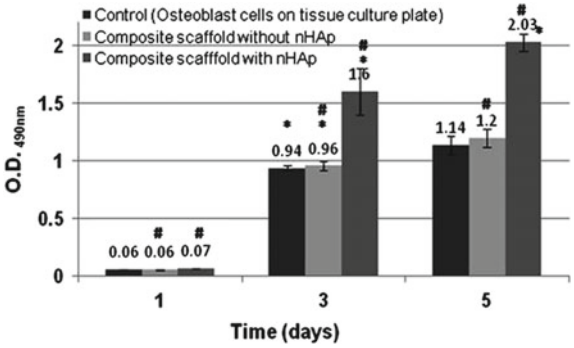

Fig. 16.1 Viability of osteoblast cells in nHAp/chitosan/gelatin/alginate composite scaffolds at 1, 3 and 5 d. Optical density is directly proportional to the cell viability. * Significant difference between control and scaffolds on the same culture day ($p < 0.05$); # Significant difference between the same scaffolds on different culture days ($p < 0.05$)

was observed in human osteoblastic cells (MG-63) when placed in direct contact with the composite scaffolds [83]. Scaffolds constructed with gelatin and chitosan were subjected to MTT assay in the presence of human fibroblast cells to check for cell viability. The results indicated that the structure of the scaffolds allowed more cells/cm^2 to grow which indicated increased cell viability and proliferation [84]. MTT assay was performed for 1, 3 and 5 d to evaluate the cell viability and proliferation of osteoblast cells over the nano-HAp/chitosan/gelatin/alginate composite scaffolds. The nano-HAp/chitosan/gelatin/alginate scaffolds showed a significant increase in cell proliferation. The better proliferation of the osteoblasts in the nano-HAp/chitosan/gelatin/alginate scaffolds was due to higher attachment of osteoblast cells on the composite scaffolds (Fig. 16.1) [85]. Thus, chitosan, gelatin, alginate, and HAp obtained from marine sources are non-cytotoxic and biocompatible and are widely used in bone tissue engineering applications.

When fabricating scaffolds for bone tissue engineering applications, they should possess cell adhesion and spread properties, which are essential for cell proliferation, differentiation, and formation of ECM. Confocal analysis revealed a large distribution of uniform MC3T3-E1 mouse preosteoblast cells on chitosan/gelatin/Laponite scaffolds after 5 d of incubation [86]. Chitosan and gelatin scaffolds stimulated bone marrow MSCs spreading, adhesion and promoted cell proliferation. Osteoblast proliferation on chitosan/alginate scaffolds was assessed using Alamar blue assay. The number of cells on the scaffolds increased up to 7 d [81]. Chitosan/gelatin/nano-HAp composite scaffolds showed the higher cell attachment and cell spreading properties. The MG63 osteoblast cells appeared flattened, sheet-like with filopodial extension [74]. The scaffolds also showed good focal adhesion and allowed cell spreading. This in turn could lead to enhanced protein adsorption and cell-material interaction. Hence, chitosan, gelatin, alginate, and HAp are promising materials for promoting cell proliferation.

16.6.1.1 ALP Staining

Alkaline phosphatase (ALP) is an enzyme which is an important marker for osteoblast differentiation during the early stages of bone formation. It plays a role in the osteoid formation and bone mineralization [87]. Total ALP activity has a clinical significance and provides good information about the bone formation and a number of active osteoblasts. Human osteosarcoma cells (Saso-2) in the presence of chitosan/HAp composite scaffolds showed high ALP activity [20, 88]. When MG-63 cells were grown on chitosan scaffolds, they had more differentiated osteoblastic behavior with increased ALP expression [46]. Apart from chitosan, gelatin, and alginate have also been proven to be ideal scaffolds for bone formation. Chitosan/gelatin/adipose-derived extracellular matrix (A-dECM) scaffolds were treated with bone marrow mesenchymal stem cells (BMSC) and subjected to ALP staining. ALP reached high levels at 7 d in the presence of osteogenic media indicating that the scaffolds displayed osteogenic ability [89]. A study involved using Silibinin-loaded chitosan nanoparticles incorporated with alginate/gelatin scaffolds with mMSCs showed enhanced ALP activity [32]. Chitosan incorporated with bioceramics have also proved to improve/promote osteoblast differentiation. The chitosan/Calcium Polyphosphate (CaPP)/0.25% Pigeonite (Pg) scaffolds were seeded with mMSCs, and ALP activity was determined at 14 d (Fig. 16.2a, b). Chitosan/CaPP/0.25%Pg scaffolds showed a significant increase of osteoblast differentiation by promoting the ALP activity [90]. In another study where MG63 cells cultured on the chitosan/gelatin/nano-silica ($nSiO_2$) scaffolds showed increased ALP activity [91]. Therefore, the scaffolds containing alginate, gelatin, chitosan or bioceramics with cells can increase ALP activity, resulting in the promotion of osteogenesis.

16.6.1.2 Alizarin Red Staining

Alizarin red stain (ARS) is derived from Anthraquinonoids family. It is commonly used in histology to highlight calcium deposits by binding it through a chelation process. Formation of alizarin red S-calcium, a birefringent compound appears red. Other compounds such as magnesium, manganese, iron can also react with ARS but are not present in sufficient concentration to cause an interaction. The HAp-loaded chitosan nanocomposite scaffolds with human fetal osteoblastic cells (hFOB) contained a significantly higher level of calcium deposition. A higher amount of mineral deposits implies a higher degree of differentiation of the cells and enhanced bone formation ability [92]. The mineral deposition on the chitosan/polypyrrole-alginate scaffolds with MG63 cells at and 14 d was confirmed using ARS. They formed apatite layer and integrated to the implant site (Fig. 16.3) [93].

Chitosan/Carboxymethylcellulose (CMC)/mesoporous wollastonite 0.5% scaffolds were studied to check if the scaffolds had the potential to promote mMSC differentiation into osteoblasts. The cells were fixed, and calcium deposits were stained. The images of the stained cells displayed a large area of mineralization [29]. Studies have been performed to improve calcium deposition by incorporating

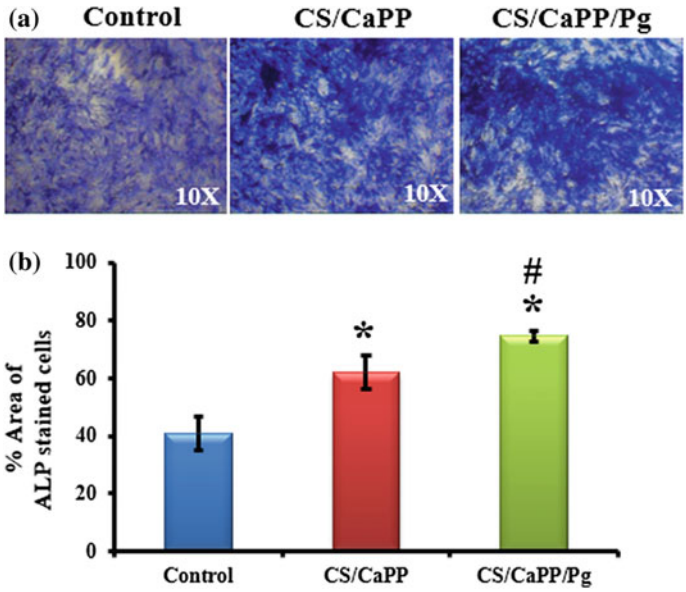

Fig. 16.2 Effect of chitosan/CaPP and Ca/CaPP/Pg scaffolds on osteoblast differentiation at the cellular level. mMSCs were treated with conditioned media obtained from scaffolds for 14 d and subjected to ALP staining. **a** Represents the light microscopic images. **b** Represents the quantified areas of the ALP staining. * Significant increase compared to control ($p < 0.05$). # Significant increase compared to CS/CaPP scaffolds ($p < 0.05$)

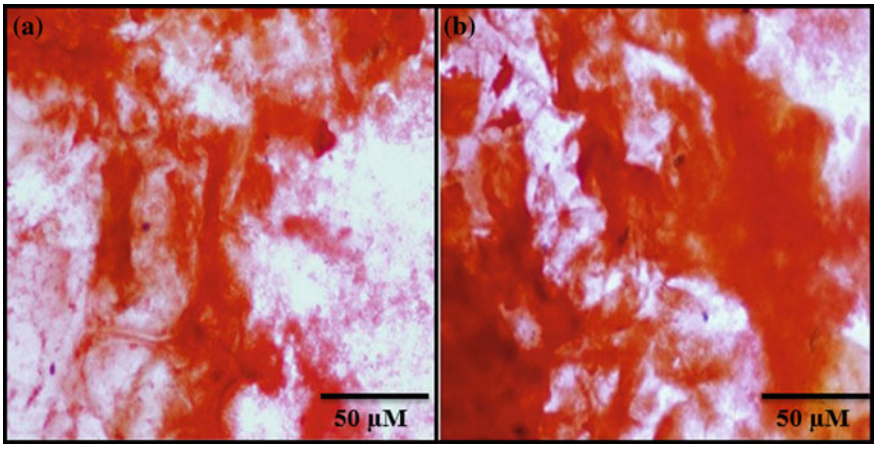

Fig. 16.3 Alizarin red staining of chitosan/PPy-alginate scaffolds incubated with cells for 7 days (**a**) and 14 days (**b**)

scaffolds with microRNAs (miRNAs). In a study, mMSCs were transiently transfected with control miRNA or miRNA-15b, followed by incubation with CMC/Zinc doped nano-Hydroxyapatite (Zn-nHAp)/ascorbic acid treated conditioned media. After 7 d treatment, cells were subjected to ARS to check for the mineralized matrix. The results indicated that there was increased calcium deposition when cells were treated with conditioned media showing the osteoinductive nature of scaffold. There was higher mineralization upon transfection of the cells with miR-15b alone. A combined treatment of both the scaffolds and miR-15b further increased the calcium deposition [94]. Scaffolds containing chitosan/gelatin-ECM were incubated with MSCs for 21 d, followed by staining. The nodules in chitosan/gelatin-ECM scaffolds appeared larger and thicker indicating calcium deposition [91]. Chitosan/gelatin/β-tricalcium phosphate (β-TCP) scaffolds were evaluated to measure the extent of matrix mineralization by human osteoblast cells for 14 d. Microscopic images showed that the calcium content on the scaffolds increased over time [78].

16.6.1.3 Von Kossa Staining

In this method, tissues are treated with a silver nitrate solution and the silver is deposited by replacing the calcium reduced by the strong light, and so can be visualized as metallic silver. The chitosan/alginate scaffolds were incubated with MG63 cells for 10 and 28 d and subjected to staining. The presence of calcium was found to appear as scattered around the cell clusters (Fig. 16.4). The mineralization was observed to increase with cell culture time. This proved that chitosan/alginate scaffolds promoted bone cell growth and mineral deposition [81]. The presence of HAp in scaffolds can improve their mineralization property. A study carried out on chiosan/HAp scaffolds showed highly mineralized nodules around osteoblasts. The calcium deposits increased with the amount of HAp present in the scaffolds [95]. Nano-HAp/collagen scaffolds were cultured for 21 d with human osteoblastic cells (hOB). A dense layer of mineralization was observed throughout the nano-HAp/collagen scaffolds [96].

16.6.2 Molecular Studies

The molecular level studies for osteoblast differentiation in vitro using reverse transcriptase-quantitative PCR (RT-qPCR) and western blot analyses provide a strong foundation to carry out studies at in vivo level for bone formation. It has been reported that many scaffolds are responsible for expression of specific genes in osteoblast cells like Runx2, ALP, Col-1, osteocalcin, etc. Runx2 is a transcription factor which regulates the expression of a number of bone forming and bone resorbing genes [97]. ALP is an early marker gene for osteoblast differentiation [87]. The late markers for osteoblast differentiation are Col-1and osteocalcin. Col-1 is one of the most abundant proteins synthesized by osteoblasts. It accounts for nearly 90% of organic bone

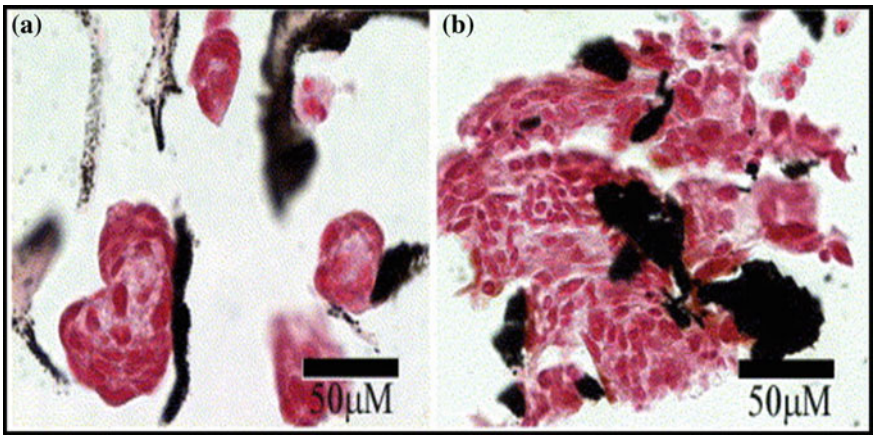

Fig. 16.4 Optical images of Von Kossa histological stain assay on chitosan/alginate scaffolds after 10 days (**a**) and 28 days (**b**) of culture in vitro

matrix mineralized by HAp [98]. Osteocalcin controls mineralization of bone, and it characterizes the mature cells of osteoblastic lineage [99]. Many molecular studies are carried out with scaffolds incorporated with bioactive molecules such as drugs, flavonoids, miRNAs to further promote osteoblastic gene expression [32, 33, 97]. For example, silibinin-loaded chitosan nanoparticles in alginate/gelatin scaffolds with mMSCs increased expression for Runx2, osteocalcin, ALP and Col-1 genes. The presence of silibinin as a flavonoid in the scaffolds further increased osteoblast differentiation [32].

Another study was carried out using nano-HAp/chitosan/gelatin/alginate composite scaffolds. High-level expression of CD44 by osteoblasts seeded over the scaffolds indicated a proper interaction between the cells and the ECM proteins present in the scaffolds. The increased expression of osteocalcin gene in osteoblasts indicated a well-maintained osteoblastic phenotype of the cells in the presence of scaffolds [85]. The chitosan/Diopside scaffolds with MG63 cells showed increased expression of ALP and Col-1 mRNAs. The osteoconductive nature of the scaffolds was evident through the upregulation of the osteoblastic differentiation marker genes in the presence of osteogenic milieu (Fig. 16.5) [89]. The mRNA levels of Runx2, ALP, Col-1 and osteocalcin in mMSCs cells were significantly increased in both 0.25% graphene oxide/chitosan/gelatin and chitosan/CaPP/0.25%Pg scaffolds [57, 90]. In 0.25% graphene oxide/chitosan/gelatin scaffolds, Runx2 protein level was analyzed by western blotting, and it showed an increased level of Runx2 protein. Therefore, these scaffolds promoted differentiation of mMSCs towards osteoblasts which was seen at the molecular level [57]. Osteoblast differentiation of CMC/Zn-nano-HAp/ascorbic acid scaffolds with mMSCs transfected with miR-15b at the molecular level was determined. The results showed that the expression levels of Runx2 mRNA and protein were significantly higher in the presence of scaffolds (conditioned media) and miR-15b when compared to an individual treatment [95]. In

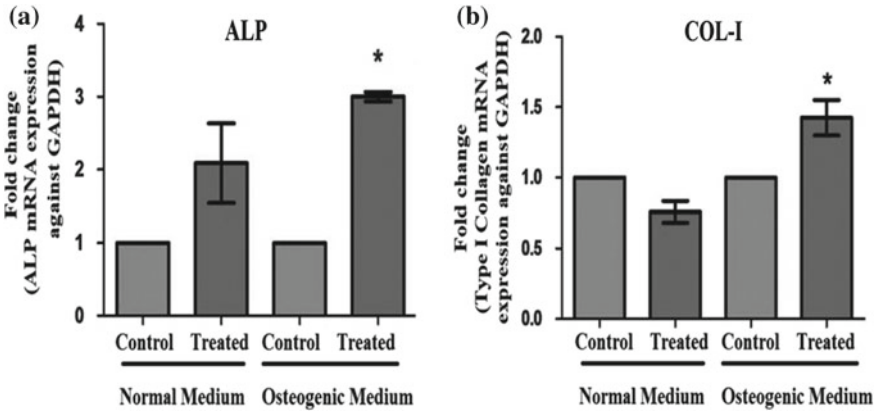

Fig. 16.5 Effect of chitosan/Diopside scaffolds on the mRNA expression of osteoblast differentiation marker genes, **a** ALP, **b** Col-I. MG63 cells were subjected to the control medium or conditioned medium (obtained from scaffolds) treatments. Cells were further grown in the presence of normal medium or osteogenic medium. * Significant increase compared to control ($p < 0.05$)

another study, the mRNA levels of ALP, Col-1, and Runx2 were assessed in mMSCs incubated with the hydrogel containing nano-HAp (Zn-chitosan/nano-HAp/β-GP) for 7 and 14 d in osteogenic media. The results showed increased expression of Runx2 and osteoblast differentiation marker genes [55]. Hence, scaffolds containing chitosan, HAp, gelatin, or alginate have the potential to promote osteoblast differentiation at the molecular level, and this effect can be further enhanced by incorporation of drugs, miRNAs into the scaffolds.

16.7 In Vivo Studies

Performing in vivo studies is crucial for the development and confirmation of novel therapies, procedures, and clinical trials. Though cellular conditions can be optimized while performing in vitro studies, it fails to replicate the precise biological condition of an organism. Therefore, to confirm these results, it is important to perform in vivo experiments using animal models. It takes into account the overall effects of a study on a living subject unlike in vitro studies. Hence, in vivo studies are necessary to prove the efficacy of the developed scaffolds for treating bone neo-formation.

16.7.1 Chitosan/Nano-hydroxyapatite

Chitosan-nano-HAp composite scaffolds can heal critical-sized defects in the calvarial rat bone. The rat calvaria is composed of cortical bone that is not highly vascularized and has a low turnover rate. Choosing a composite scaffold from marine

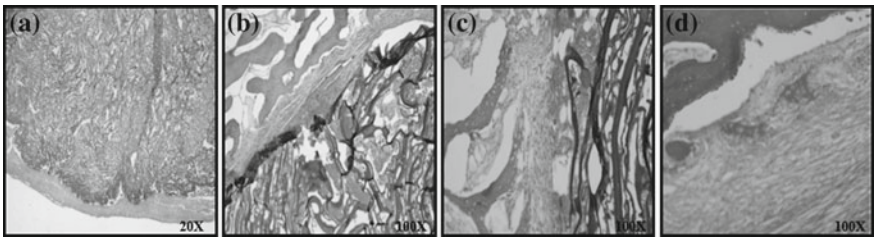

Fig. 16.6 Microscopic images of new bone in the defective area of rabbit tibia at 8 week of chitosan/nano-HAp transplantation at (**a**) 20X, (**b**) 100X magnifications. **c** Microscopic images indicated the bony defect with osteoblasts. **d** The marginal defective region showed cells associated with ossification, bone trabeculae and a few islands of ossification

sources can contribute to limiting scaffold degradation thus promoting bone growth [100]. When chitosan/nano-HAp scaffolds incorporated at the defective region of rabbit tibia, there were signs of focal ossification and adherence of the implanted material to the original bone. New bone formation appeared after 8 weeks transplantation of chitosan/nano-HAp scaffolds as shown in Fig. 16.6a, b. The biomaterials along with osteoblasts, were seen in the defective region when examined (Fig. 16.6c). The marginal region of the defect was then focussed which showed bone trabeculae and cells associated with ossification (Fig. 16.6d). It has therefore been concluded that chitosan/nano-HAp scaffolds are the effective composite scaffolds with great potential in the field of bone tissue engineering [101]. The bone-healing property of the Zn-chitosan/nano-HAp/β-GP hydrogel was assessed in rats with tibial defects. Radiographic analysis was taken at 2 week post-surgery. The results showed better tissue organization in the presence of hydrogel. It also showed wound healing and bone formation property due to the presence of nano-HAp in the hydrogel [55]. Micro-computed tomography is suitable for analyzing three-dimensional microstructure of bone. Silk-fibroin/chitosan/nano-HAp scaffolds exhibited neo-tissue formation, cartilage and subchondral bone formation at the peripheral areas. Upon dissection, layers were shown to be sufficiently repaired whereas the vacant region at the center disappeared rapidly (Fig. 16.7) [102].

16.7.2 Chitosan/Gelatin

The in vivo studies using chitosan/gelatin scaffolds showed an increase in bone tissue growth and regeneration. Examination of newly formed bone by Van Gieson's picrofuchsin staining using light microscope at 8 wk supported the above result. Chitosan/gelatin treated defects had higher cartilaginous, and bone cells and the healing were more advanced. The histopathological features of chitosan/gelatin scaffolds revealed newly formed cells proliferating from hypertrophic bone edges into the middle portion of the defect area and connective tissue, resulting in the formation

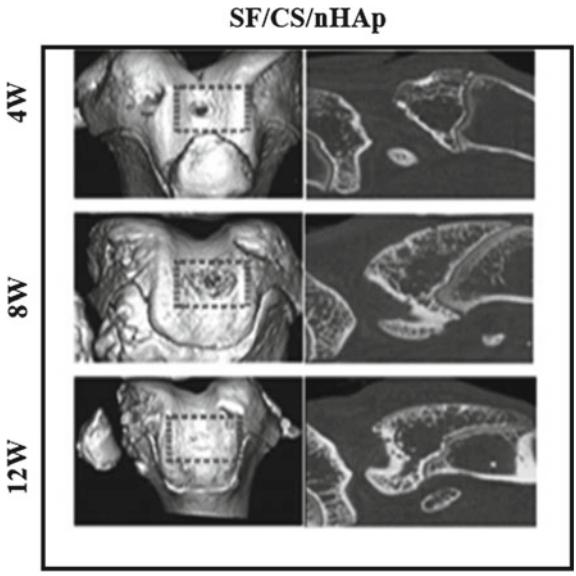

Fig. 16.7 Micro-CT images of silk-fibroin/chitosan/nano-HAp of implants showed new tissue formation in tibia of the rabbit

of cartilaginous tissue (Fig. 16.8a) [103]. Another combination of scaffolds that consisted of chitosan/gelatin/nano-HAp/nano Cu-Zn in rabbits showed new tissue infiltration and large number of cells in the matrix. A granulation tissue grew inside the chitosan/gelatin/nano-HAp/nano Cu-Zn scaffolds which indicated that the scaffolds provided a vascularized network for deposition of collagen and tissue regeneration [104]. A study with rat tibial bone defect model system was carried out to determine the role of fabricated graphene oxide/chitosan/gelatin scaffolds in vivo. The radiographs of the rat tibial defect area were taken after 2 week and examined (Fig. 16.8b). There was improved wound closure, and an alteration in the area of bone defect changed to oval morphology which suggested bone healing. Bridging of the defect area as well as new bone growth due to scaffolds suggested that the scaffold is biocompatible and osteogenic [57].

16.7.3 Chitosan/Alginate

It was reported that the chitosan/alginate scaffolds promoted rapid vascularisation, deposited connective tissue and calcified matrix within the entire scaffold structure [81]. Tissue compatibility of chitosan/alginate scaffolds infused with bone marrow was assessed in vivo by implanting them into muscles of rats and harvesting them at different time periods of implantation. There was no evidence of infections or complications reported. The harvested scaffolds were stained with Masson's to visualize the formation of collagen and vascularization. It was found that collagen was deposited throughout the scaffolds, indicating that the cells have fully penetrated

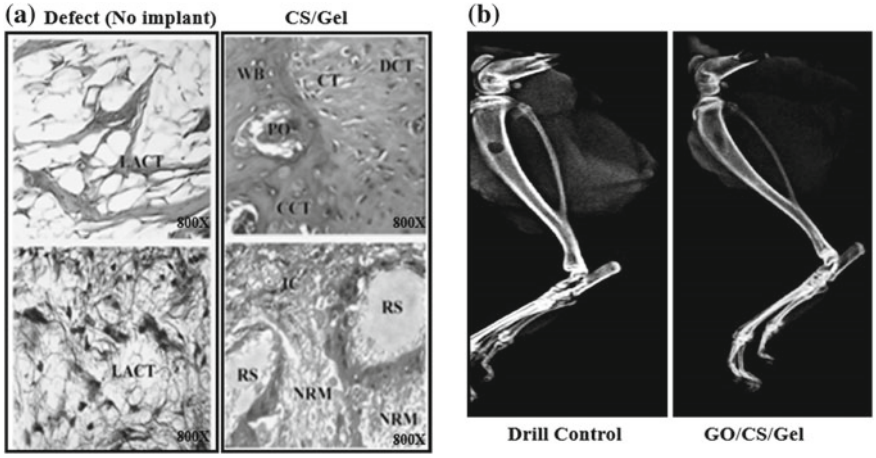

Fig. 16.8 **a** Histopathological staining of the radial bone defects (H&E Stain). Loose areolar connective tissue and cartilaginous cells appeared in the lesion of defect group. Mild inflammatory reaction and connective tissue were seen in the defects treated with the chitosan-gelatin along with the remains of the scaffolds. (RBE: radial bone edge; LACT: loose areolar connective tissue; DCT: dense connective tissue; CZ: cartilaginous zone; WB: woven bone; CCT: calcified cartilaginous tissue; IC: inflammatory cells; RS: remnants of scaffold; FCZ: fibrocartilaginous zone; CT: cartilaginous tissue; FCT: fibrocartilaginous tissue; CZ: cartilaginous zone; PO: primary osteon; NFWB: newly formed woven bone; RBCs: red blood cells; NRM: newly regenerated matrix). **b** Healing of rat tibial bone defect. X-ray images of the rat tibial bone defects of control and 0.25% graphene oxide/chitosan/gelatin scaffolds after 2 week post implantation

into the scaffolds (Fig. 16.9a, b). At 4 week, it was observed that a large number of small blood vessels were seen in the scaffolds (Fig. 16.9c) and the size of the blood vessels increased over time from 4 to 12 week (Fig. 16.9d). The results showed a high angiogenic response of the host to the implanted construct which ensured that there is sufficient nutrient flow in the scaffolds which allowed all types of cells to survive and proliferate for extended periods. Thus, the results above indicated the potential applications of chitosan/alginate scaffolds as an ideal alternative for bone tissue engineering applications.

It was also proven that HAp/chitosan/alginate composite scaffolds act as bone substitute materials which could promote cell attachment and proliferation. Micro-CT images of a mouse skull showed initial mineralization in the area of the bone defect which indicated that the site implanted with the composite scaffolds started healing with a new generation of the bone [105]. The study with the HAp/chitosan/alginate composite scaffolds also confirmed to be more effective for new bone generation by performing H&E staining through implantation experiments. The chitosan/Diopside scaffolds were assessed for their ability of bone formation in vivo [89]. The results showed that the neutrophils were present at the periphery of the scaffolds which was observed by the H&E staining. Trichome staining showed deposition of collagen onto the chitosan/Diopside scaffolds aiding in cells recruitment, attachment,

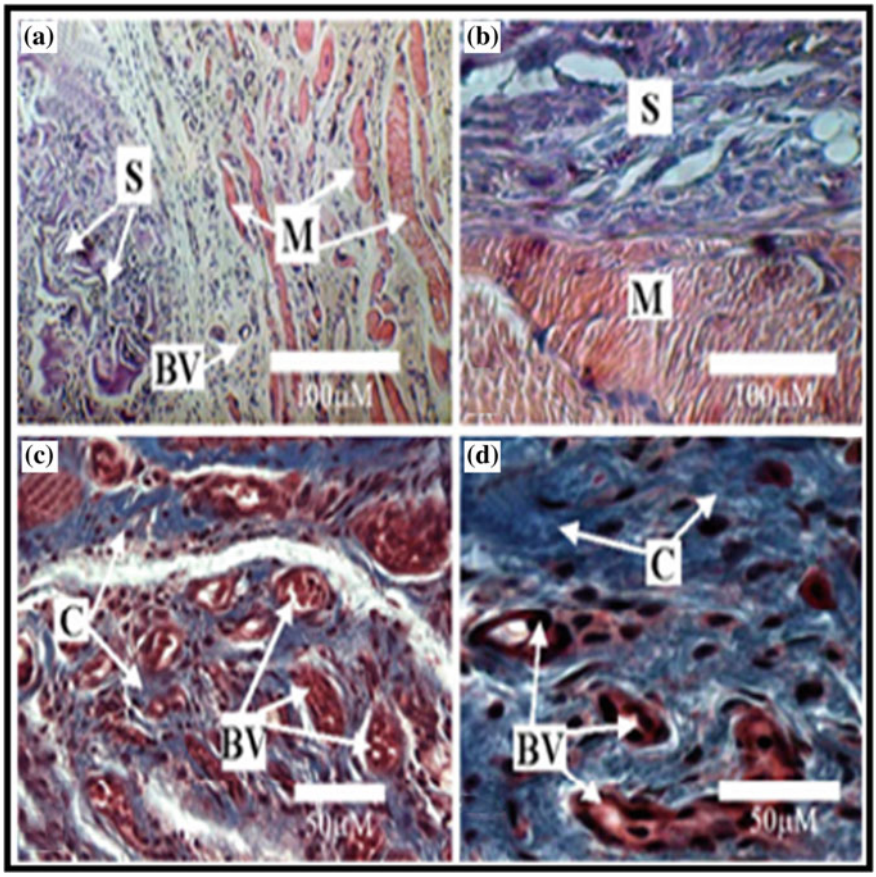

Fig. 16.9 The optical micrograph images of the stained scaffolds in trochanteric area of the rat for bone marrow (**a**) and (**b**). **c** H&E Stained images showed no evidence of fibrotic layers and was well integrated with the tissue (at 12 week). **d** Masson's trichrome stained images at week 4 showing the formation of a large number of blood vessels (BV) and collagen deposition (**d**). (S: scaffold; M: muscle; C: collagen; B: blood sinus; BV: blood vessels)

and proliferation. Chitosan/CaPP/0.25%Pg scaffolds were checked for their effect on the critical-sized bone defect of rat tibia. H&E and Masson's trichome staining were performed. The results showed the well-distinctive structural characteristics of bone, bone marrow elements, bone marrow spaces along with mature bone/osteoid and new areas of bone formation [55].

16.8 Conclusions

Marine-derived materials constitute a promising source of unique scaffolds which have considerable pharmaceutical and therapeutical potential. This chapter highlighted the materials such as chitosan, gelatin, alginate, and HAp obtained from marine sources and their potential applications towards bone tissue engineering applications as biocomposite scaffolds. These scaffolds significantly improved their mechanical properties such as tensile and compressive strength. Also, the in vitro and in vivo studies involving these biocomposite scaffolds for bone tissue engineering were discussed. The in vitro studies have proved that the biocompatibility and osteoblast differentiation at the cellular and molecular levels were supported by the scaffolds. The in vivo studies have confirmed the role of scaffolds in promoting bone tissue regeneration using the animal model system. These outcomes suggested that in general, most of the synthesized marine source based biocomposite scaffolds have the potential to be used in tissue engineering, dental surgeries, drug delivery applications.

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Chapter 17
Calcified Algae for Tissue Engineering

Gina Choi and Louise A. Evans

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

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

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

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

17.1 Biomineralization

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

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

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

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

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

17.2 Bone

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

17.2.1 Mineral Phase

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

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

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

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

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

Table 17.3 Selected crystal classes of calcified systems

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

17.2.2 Organic Phase

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

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

17.2.3 Bone Architecture

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

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

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

17.2.4 Bone Remodelling

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

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

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

17.2.5 Bone Grafts

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

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

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

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

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

17.2.6 Bone Substitute Materials

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

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

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

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

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

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

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

17.2.7 Bone Tissue Engineering

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

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

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

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

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

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

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

17.3 Calcium Phosphate Bioceramics

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

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

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

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

17.3.1 Synthesis Methods

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

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

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

Table 17.5 Synthesis methods used to produce HAp

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

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

17.3.2 Synthesis from Biogenically Derived Minerals

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

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

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

17.3.2.1 Coral

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

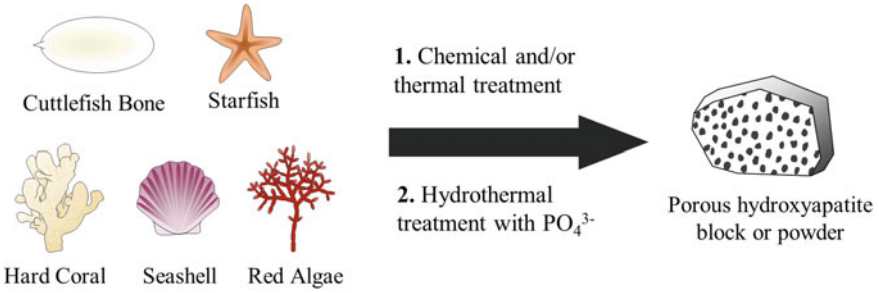

Fig. 17.1 Schematic diagram showing synthesis of porous or powdered HAp bioceramics derived from marine exoskeletons. Step one is the removal of organic material and step two is the conversion of calcium carbonate biominerals to HAp

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

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

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

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

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

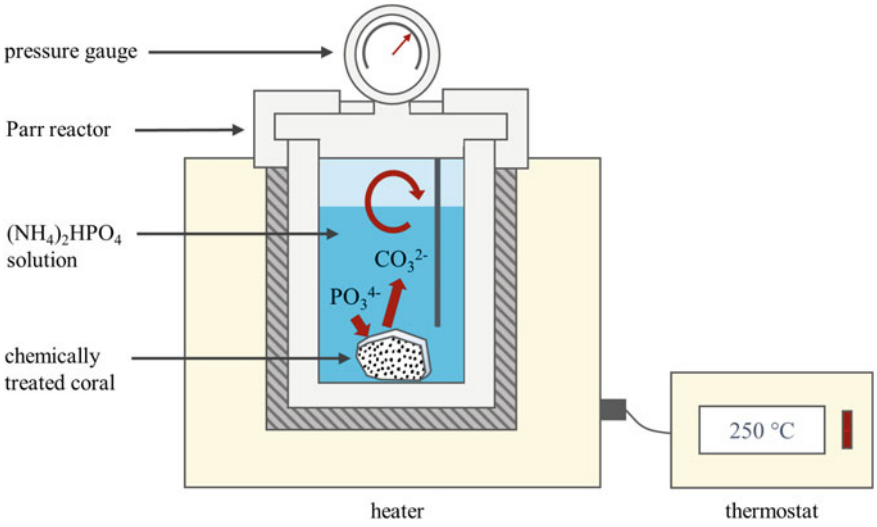

Fig. 17.2 Diagram of the hydrothermal apparatus typically used to convert aragonitic coral to HAp. The sealed Parr reactor is subjected to a high temperature which increases the pressure within the reactor. As a result, phosphate ions from the solution exchange with carbonate ions in the coral structure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

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

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

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

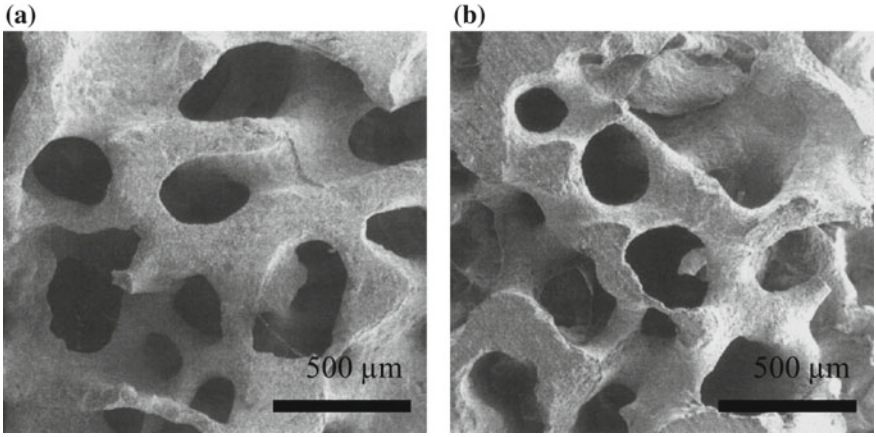

Fig. 17.3 SEM micrographs of Australian *Goniopor*a coral. **a** Prior to conversion; and **b** after hydrothermal conversion demonstrating the preservation of interconnecting pores (adapted from [70])

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

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

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

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

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

17.3.2.2 Calcified Macroalgae

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

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

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

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

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

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

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

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

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

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

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

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

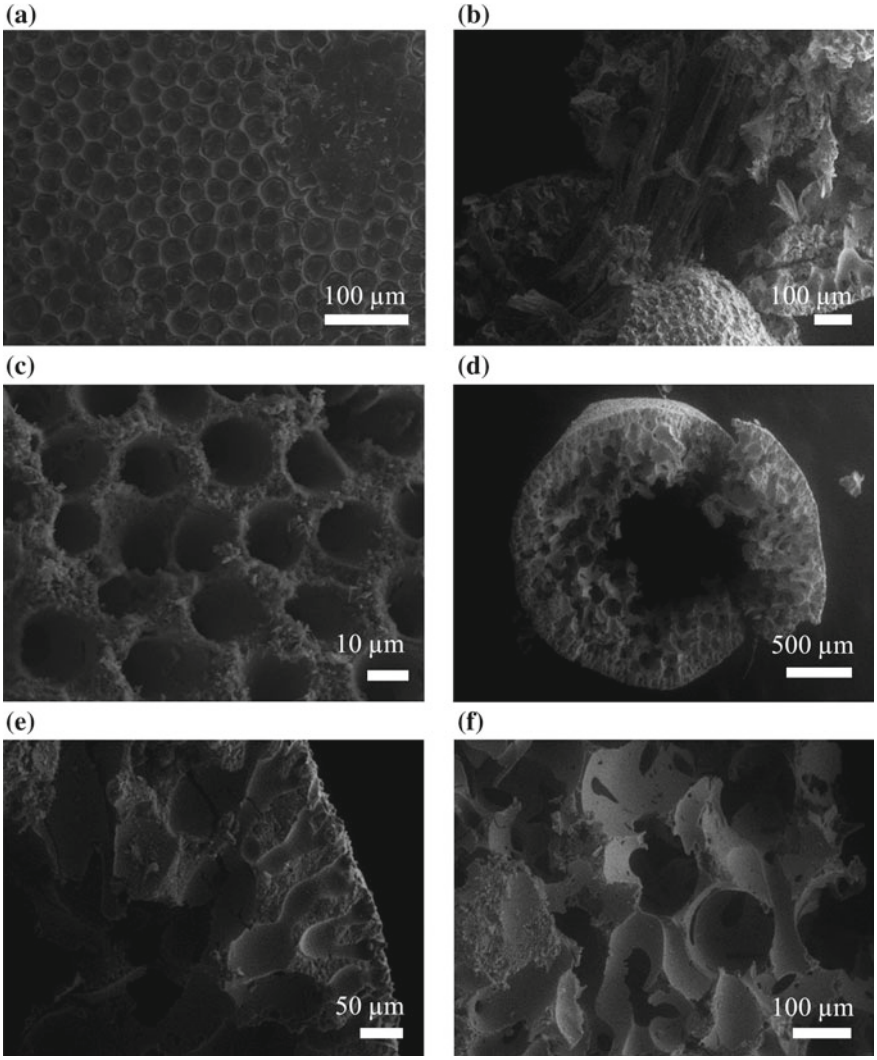

Fig. 17.5 SEM micrographs of green algae *H. cylindracea* **a** surface after cleaning; **b** longitudinal cross-section after cleaning; **c** surface after conversion; **d** and **e** transverse cross-section after conversion, and **f** inner surface after conversion

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

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

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

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

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

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

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

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

17.5 Techniques for Analysing Biogenically Derived HAp Materials

17.5.1 Fourier Transform Infrared Spectroscopy

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

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

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

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

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

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

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

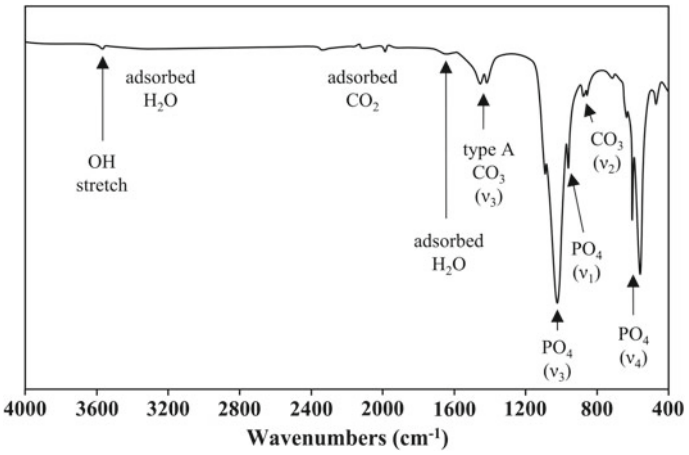

Fig. 17.6 FTIR spectrum of HAp derived from green algae *H. cylindracea* (Choi, unpublished data)

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

17.5.2 X-ray Diffraction

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

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

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

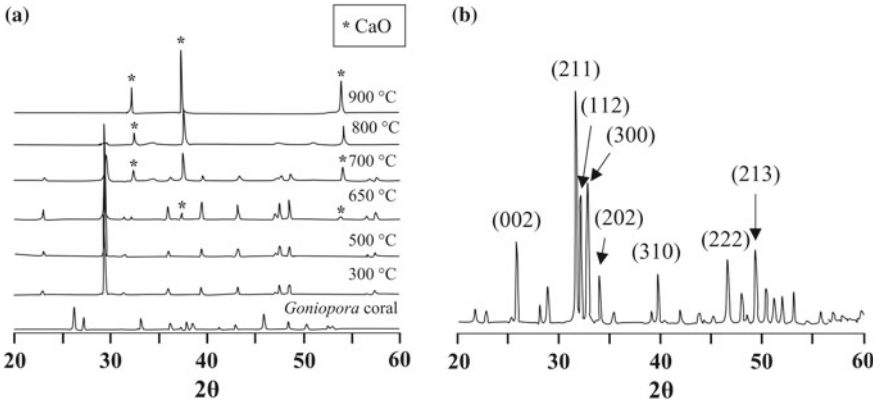

Fig. 17.7 XRD patterns of **a** *Goniopora* coral with heat treatment at various temperatures resulting in the phase transformation of aragonite to calcite then CaO and **b** coralline HAp with significant Miller indices (adapted from Hu et al. [70])

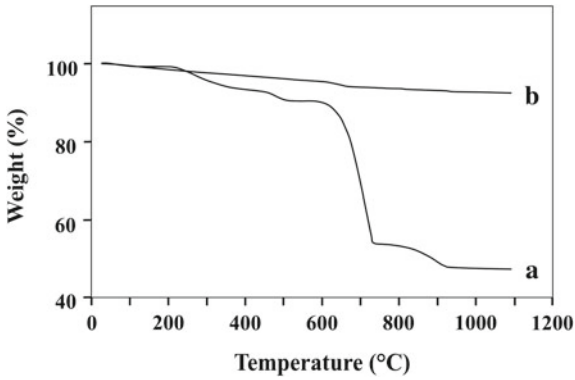

Fig. 17.8 TGA traces of **a** calcified algae *H. cylindracea* and **b** *H. cylindracea* derived-HAp (Choi, unpublished data)

17.5.3 Thermogravimetric Analysis

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

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

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

17.5.4 Scanning Electron Microscopy

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

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

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Chapter 18
Chitosan-Based Biocomposite Scaffolds and Hydrogels for Bone Tissue Regeneration

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Abstract Natural biomaterials derived from marine sources are gaining attention in the field of bone tissue engineering owing to their biodegradability, biocompatibility, bioactivity, and structural similarity with the natural bone extracellular matrix. They recapitulate bone microenvironment and components of natural bone tissue which augments treating critical-sized bone defects. Negating the necessity of revision surgeries due to its biodegradable nature, marine biomaterials based biocomposite scaffolds provides various advantages over the conventional routes of employing metallic implants for bone tissue repair and regeneration. Marine biota provides renewable resources for isolation of biopolymers such as chitosan, alginate, collagen, fucoidan and hydroxyapatite bioceramics that are widely explored for its biomimetic properties in bone tissue engineering. In this chapter, we explore the role of composites fabricated using biomaterials isolated from the marine source, especially chitosan for bone tissue regeneration.

Keywords Chitosan · Marine biomaterial · Biocomposites · Osteoblasts · Bone tissue engineering

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18.1 Introduction

Bone is a highly vascularized connective tissue and undergoes continuous, precise remodeling during the lifespan of an organism [1]. Bone forms the structural frame work of the skeletal system and is involved in locomotion, housing bone marrow, encasing internal organs and mineral homeostasis [1, 2].

Critical-sized bone defects (CSBD's) adversely affect the functions of bone which often requires external augmentation for correcting bone defects and promoting bone healing [3]. Grafting procedures harness bone from living donors, deceased patients or from other species to treat bone defects. Despite various grafting procedures, autografting is still considered to be the gold standard method for treating CSBD's owing to the osteoconductive and osteoinductive properties. Restrictions in the amount of tissue to be harvested, donor site morbidity, immune rejection, and risk of pathogen transfer are various setbacks associated with traditional grafting strategies [4–6].

The utilization of metallic implants is another common approach that has been used extensively as a number of surface modification techniques can be applied to improve its bioactivity and to enhance the osseointegration process [7–11]. Despite, the surplus availability of implant materials, the necessity to perform revision surgeries to remove implanted material due to its non-biodegradable nature, post-operative surgeries associated with pains and infections needs to be seriously addressed using various strategies. Bone tissue engineering adjoins the principles of bone biology and tissue engineering to treat bone loss through the involvement of (a) biodegradable temporary matrices (scaffolds), (b) stem cells expanded ex vivo and (c) combination of both biomaterial constructs and cells [12–16].

Biomaterial-based tissue engineering approach provides an amenable environment for cell growth and offers viable tissue equivalents by combining the orchestra between biomaterials and cells. Cellular growth is directed through the use of biomaterials (polymers, ceramic particles, and metallic nanoparticles), polymeric constructs offers unique advantages of recreating and mimicking the three-dimensional (3D) extracellular matrix microenvironment [17–21]. A number of studies have shown that strategies involving biomaterials offers unique advantages such as biodegradability during bone formation, biocompatibility, tunable properties and freedom to choose from a wide range of polymers with specific characteristics.

The use of preformed scaffolds and hydrogels are widely explored by either fabricating it with a single polymer or as a composite with multiple polymers substituting one or other missing properties required for efficient bone regeneration. Scaffolds are three-dimensional matrices with a porous architecture capable of supporting bone healing and allow new bone tissue ingrowth [22–24]. Scaffolds are either preformed or often sculptured to gear a geometry matching the bone defect. Hydrogels are three-dimensional hydrophilic matrices of crosslinked polymeric chains capable of absorbing several folds of water to mimic host tissue microenvironment [25, 26].

Choosing the correct polymeric material is vital in determining the success of any biomaterial-based bone graft irrespective of whether scaffolds or hydrogels are used to promote bone regeneration. Natural and synthetic polymers have been investigated

due to their ability to promote bone formation and as a carrier of cells/bioactive molecules that will aid bone regeneration. Amongst them, natural polymers such as chitosan, alginate, collagen, gelatine, silk fibroin and glycosaminoglycans showed great promises due to their biocompatibility and structural resemblance with natural extracellular matrix components [21, 27]. These properties generate a less hostile environment for the recruited cells to multiply and differentiate.

18.1.1 Bone and Its Components

Bone is classified into trabecular and cortical bone, and it is highly vascularized with orchestrated three-dimensional structural components at macro, micro, and nano dimensions. The basic extracellular matrix component of bone constitutes collagen fibrils. These collagen fibrils are anisotropically arranged to provide adequate mechanical strength and deposits hydroxyapatite (HAp) crystals. Osteons are the repeated units which constitute cancellous bone containing concentric bundles of collagen nanofibers called as lamellae arranged around a central canal. Trabecular bone is filled with free spaces to encase bone marrow and enormous vasculature. Calcium phosphate crystals are deposited along the axis of collagen and both with organic proteins thereby creating a mechanically stronger and functional syncytium. Osteoblasts, bone-forming cells and, osteoclasts are bone resorbing cells maintains a precise balance through a process of bone remodeling. Disruption in this high precision leads to pathologies that reversibly or irreversibly affect the structure and functions of bone [1, 2].

18.1.2 Marine Biomaterials for Bone Tissue Regeneration

Natural biomaterial-based composites are widely investigated as preformed scaffolds and injectable hydrogels for bone tissue engineering applications. Implications of natural biomaterial-based bone grafts possess various advantages over their counterparts, synthetic materials. Marine biota is rich in biodiversity with a surplus supply of resources that are largely exploited for biomedical applications. Biomaterials isolated from marine sources include (a) chitosan-isolated from crustaceans, (b) alginate and fucoidan from sea weeds, (c) collagen from fishes and (d) hydroxyapatite from corals and shelled organisms.

These biomaterials including polymers and ceramics are utilized to fabricate bone tissue engineering matrices either used as a stand-alone material or combined to form a composite material with other polymers/ceramics/nanomaterials. Our research group extensively deals with chitosan-based composites for bone tissue engineering applications as scaffolds, and hydrogels. Hence, in this book chapter, chitosan and its composites role in bone tissue engineering are elaborated citing few of our interesting composites.

18.2 Chitosan

Chitosan is the deacetylated product of chitin, isolated mainly from the crustacean shells including crab, shrimp, lobster, and corals. Crab and shrimps are the major sources for the chitosan production. Chitosan (Fig. 18.1) is a copolymer consisting repeating units of D-glucosamine and N-acetyl-D-glucosamine linked by β-(1-4) glycosidic linkages [28].

Over the past two decades, chitosan has been explored considerably for its potential application in biomedicine. Chitosan is being investigated for applications in tissue engineering and drug delivery, owing to its biocompatibility, biodegradability, ability to undergo chemical modifications, exhibits porous structure, suitability to support cell adhesion and growth, osteo conduction and antibacterial activities [29]. Chitosan is used in fabricating bone tissue engineering grafts in two forms (i) preformed scaffolds, and (b) hydrogels. Chitosan is either used as a stand-alone polymer or as a composite with other polymers to enhance the properties of chitosan. Anionic polymers, ceramic particles, nanoparticles, bioactive molecules, and cells are added in combination with the naive chitosan polymeric matrix to facilitate effective bone regeneration [21]. In addition, chitosan is capable of being modified using a variety of approaches to produce "modified chitosan", which is utilized for bone regeneration with improved properties.

18.2.1 Physicochemical Properties

If the number of N-acetyl glucosamine (NAG) units exceeds 50%, then it is referred to as chitin; on the other hand, if the number of N-glucosamine units is greater than 50%, then it is said to be chitosan. The variation in the degree of acetylation (50–95%) generates various forms of chitosan with molecular weights ranging from 300 to 1000 kDa [28, 30]. Physicochemical properties of chitosan such as solubility, degradation, stability, and solubility are highly dependent on the degree of acetyla-

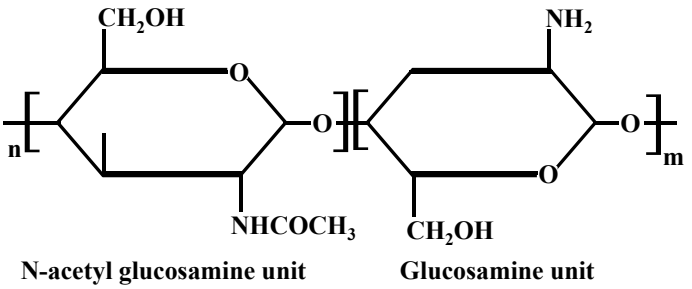

Fig. 18.1 Structure of chitosan. Chitosan consists of repeated units of N-acetyl glucosamine (NAG) and Glucosamine

tion. The solubility is determined by the amount of free amino and N-acetyl groups presence in chitosan structure. Chitosan is soluble in acidic pH with pKa of 6.5, and insoluble in neutral and basic solutions [28].

Once chitosan is completely dissolved, the free amine groups are protonated which imparts a positive charge to the chitosan chains. The intensity of positive charges increases with an increase in the degree of deacetylation. This cationic property is very crucial for its application in bone tissue engineering as chitosan can form polyelectrolyte complexes with anionic macromolecules. The properties of chitosan can be adjusted through various modification approaches such as the addition of functional groups in the hydroxyl groups present at C2, C3 and C6 positions, an amino group and linear polyamine [31].

Chitosan can be easily adjusted and modified to form films, sponges, fibres, beads, nanospheres, and other complex geometries for orthopaedic applications. Chitosan is targeted by Lysozyme, an enzyme that degrades chitosan in vitro and in vivo via the hydrolysis of NAG-NAG, NAG-glucosamine, and glucosamine-glucosamine linkages. The degradation of chitosan chains is inversely proportional to the degree of deacetylation. Thus, it is possible to control the in vivo degradation of chitosan to match the progress of bone tissue ingrowth [31].

18.2.2 Biological Properties

Chitosan possesses many vital properties that make it an ideal candidate for bone tissue engineering applications. Chitosan provokes a minimal immune response and fibrous encapsulated upon in vivo implantation [32]. Chitosan-based matrices have shown to accelerate wound healing by modulating immune response especially neutrophils, macrophages, and fibroblasts [33–36]. The cationic nature of chitosan attributes to its interesting biological properties. It has been shown that chitosan promotes blood clotting through its hemostatic action by binding to negatively charged red blood cells. Consequently, they are used to generate various wound dressings [37, 38]. The cationic nature of chitosan imparts antibacterial properties against both gram-positive and gram-negative strains. The mechanism behind its antibacterial action is not completely understood; however, it has been proposed that the bacterial cell wall is most likely to be disrupted by chitosan. Furthermore, it is believed that chitosan is also responsible in disturbing the biosynthesis process and inhibits mass transports across the cell walls [39]. In addition to these properties, the amine and hydroxyl groups of chitosan chains are exploited for applications in drug delivery owing to the mucoadhesiveness exerted by chitosan. Adherence of chitosan to mucosal surface result in sustained drug delivery and adsorption which increases the bioavailability and half-life of various drugs [40].

Chitosan also supports protein adsorption and cell adhesion due to the hydrophilic properties and hence it provides an amenable environment for cell growth. In vitro studies demonstrated that chitosan promotes cellular interaction, proliferation and differentiation of mesenchymal stem cells, osteoblasts, and induced pluripotent stem

cells [41–44]. Chitosan is found to be osteoconductive in nature and promotes osteoblast differentiation in the presence of osteogenic stimulants [42].

18.2.3 Routes for Fabricating Chitosan-Based Scaffolds and Hydrogels

Bone tissue regeneration requires 3D matrices to support the growth of osteoblasts, differentiation and subsequent bone formation. Bone growth and remodelling in vivo are orchestrated by multiple cell types in a 3D microenvironment. Hence, it is important to mimic the tissue microenvironment in order to achieve maximum bone regeneration. Chitosan can be moulded into various geometries, and thus, 3D scaffolds are produced with ease using various fabrication methods which include (a) lyophilization, (b) free gelation, (c) solvent leaching, (d) electrospinning, and (e) 3D printing [45, 46].

Lyophilization involves the basic principle of sublimation where the solvent is frozen; ice crystals are formed and sublimated to produce pores in the original space occupied by the solvent crystals. High porous interconnectivity is usually observed in the scaffolds produced by lyophilization. The collapse of pores due to minor changes in the temperature is one of the major drawbacks of this technique. Solvent-exchange or phase separation method employs an alkaline solution below the gelation point of the polymeric solution. This method also generates enough porosity to the scaffold but lacks mechanical strength. Controlling the pore size and dimensions is achieved by salt leaching method. Incorporating sodium chloride crystals of defined shape and size as porogens into chitosan matrix and leaching by using water to from pores in the structures is followed in this method.

Electrospinning allows the fabrication of scaffolds fibrous architecture with control over the diameter of the fibres. It utilizes electric field between the nozzle tip of the polymeric reservoir and rotating ground collector. Electrospinning is a versatile technology in encapsulating various drugs, biomolecules which are sensitive to pH and temperature [45, 46]. Multiple polymeric fibres can be obtained in a single sheet of electrospun membrane.

Although the previously discussed methods possess various advantages, controlling the scaffold architecture matching the bone defect geometry is usually not achievable. Reproducible geometries exactly matching the defect dimensions is usually generated by rapid prototyping. It involves computer-aided design data to fabricate 3D scaffolds with desired architecture.

Apart from scaffolds, chitosan has also become increasingly attractive in the form of injectable hydrogels for bone tissue engineering applications due to the minimal invasiveness associated with chitosan hydrogels. Thermosensitive hydrogels have promising properties of gelation at physiological temperature (37 °C) and neutral pH which assists in loading hydrophilic, pH-sensitive drugs, growth factors and live

cells. Temperature dependant gelation is achieved by the controlled addition of polyol salt, majorly β-glycerophosphate [47].

18.3 Chitosan Scaffolds for Bone Tissue Regeneration

By imitating the bone architecture and composition, various scaffolds have been investigated in the field of bone regeneration. An ideal scaffold intended for bone tissue engineering should exhibit various properties which include (i) porous architecture, (b) water adsorption, (c) protein adsorption, (d) biocompatibility, (e) biodegradability-matching host bone tissue ingrowth, (f) mechanical properties, (g) biomineralization, and (h) ability to interact with cells.

Although chitosan possesses various properties that enables its candidature for bone tissue engineering applications, lack of adequate mechanical strength, the absence of osteoinduction, and poor biomineralizing ability has limited its use as a single standing polymer. Hence, along with chitosan other polymers, ceramics, bioactive molecules are included to improve the properties of chitosan-based biocomposites suited for bone regeneration [17, 18, 21].

18.3.1 Porosity

Pores in the scaffolds are necessary to promote cell infiltration, growth, secretion of extracellular matrix (ECM) and new tissue ingrowth [48]. In addition, nutrient diffusion and metabolic waste elimination are also made possible by the presence of pores. Pore geometry and dimensions govern the functional outcome of the fabricated scaffold. Smaller pores limit cell permeability and induced capsule formation around the scaffold edges. In contrast, larger pores decrease the mechanical properties and reduce the ligands density necessary for cellular interaction [49]. Therefore, precise control in the pore sizes is crucial for maintaining optimal cellular interaction and forming functional tissue graft upon implantation in vivo [50, 51]. A number of investigations has suggested that pores ranging between 20–100 μm is ideal for cells to infiltrate, while pores greater than 100 μm in size will favours toward neovascularisation. Pore dimensions greater than 300 μm have been assumed to lead to direct osteogenesis in scaffolds [52]. Chitosan scaffolds exhibit pores, and it is controlled by its concentration, the addition of secondary polymers/ceramic particles/nanoparticles, cross-linkers percentage and fabrication conditions.

18.3.2 Swelling Property

Water retention property of the scaffolds is crucial in tissue engineering to support cell adhesion and tissue ingrowth. Scaffolds absorb considerable amounts of fluids from the nearby tissues and expand with an increase in the overall surface area and pore sizes. Increased pore size avails cells to penetrate deep into the scaffolds and improves ECM deposition. The increased swelling property would eventually disrupt the porous structure, implant loosening and retraction from the implanted region. Similarly, a decrease in swelling would ultimately reduce cell interaction and hinders tissue ingrowth. Therefore, an optimal amount of swelling is necessary for efficient bone tissue regeneration. Chitosan chains are hydrated by the protonation of amine/imine groups in contact with water and results in mechanical relaxation of the tightly coiled chains of chitosan. Amino groups of chitosan participate in electrostatic interaction with oppositely charged polymers remarkably decreases the swelling property [21, 26]. Control over the swelling rate of chitosan is achieved by the controlled addition of electrostatically opposite polymers, crosslinkers and other reactive nano-particles [53].

18.3.3 Protein Adsorption

Adsorption of proteins is a pre-requisite for cellular interaction with the biomaterial. Tissue engineering scaffolds upon in vitro exposure to cell culture medium or upon in vivo implantation have the ability to rapidly absorb proteins on their surface. Proteins adsorption initiates cell spreading, proliferation and differentiation. Cells interact with the absorbed protein via integrins and induce cytoskeletal remodelling resulting in cellular functions [54]. Amino groups and carboxyl groups of chitosan has been known to interact with proteins via electrostatic forces and van der Waals interaction [55]. Strategies to improve the mechanical strength of chitosan scaffolds based on the addition of other polymeric materials or via crosslinking may greatly reduce the protein adsorption capabilities due to the limited availability of reactive amino, hydroxyl and carboxyl groups. Hence, in an effort to improve protein adsorption capacity, the inclusion of various nanoparticles as well as the addition of protein sequences and functional group modifications have been investigated [17, 18, 21, 42].

18.3.4 Biomineralization

Deposition of minerals and ions on the surface of the implanted scaffold upon its exposure to body fluids is known as biomineralization. Biomineralization property greatly improves the osseointegration of implanted graft in vivo. Human bone possesses ~70% of inorganic crystals comprising of HAp, and therefore, scaffolds

with biomineralizing ability provide interfacial bonding with host bone and the implant [56].

Chitosan is known to possess biomineralizing ability. In general, an increases in certain reactive groups will lead to an improvement in the amount and rate of mineral deposition to form bone-like apatite with a Ca/P ratio of 1.6 [57]. This could be achieved by including nanoparticles, ceramic particles, graphene oxide sheets, carbon nanotubes and chitosan modifications [18, 58, 59].

18.3.5 Biodegradation

Degradation of the implanted biomaterials should match the rate of new bone formation within the scaffold. Exposure of biomaterial scaffold to body fluids results in gradual break down of the polymeric units by hydrolytic enzymes. The degraded product is generally incorporated into various metabolic pathways thereby minimizing the risk of toxicity and does not provoke an immune response. The long-term success of the implanted biocomposite scaffold is dictated by its ability to degrade with no toxic by-products and simultaneously promotes bone formation [60].

Predominantly, the degradation of chitosan is due to the actions of circulating lysozyme. Hydrolytic scission of the polymeric units of chitosan releases degraded products (amino sugars) which are incorporated into glycoprotein metabolic pathways [61]. A higher degree of deacetylation will produce a quicker rate of degradation of chitosan. The degradation rate of chitosan can be controlled by limiting the amount of free functional groups available, electrostatically complexing with other polymers and inclusion of cross linkers.

The following section provides a brief discussion on various chitosan-based biocomposites synthesized in our laboratory. In-depth understanding into the physicochemical and biological properties of the scaffolds is also provided in this section.

18.4 Chitosan-Based Biocomposite Scaffolds for Bone Tissue Regeneration

18.4.1 Chitosan/Nanohydroxyapatite/Nano-Silver Composite

Chitosan, when used as a stand-alone polymer, lacks osteoconductivity which limits its application as grafts in most of the critical-sized bone defects. Despite aseptic surgical procedures, bacterial infections are common and, in some cases, leads to implant failure [62, 63]. To address the aforementioned drawbacks, we fabricated and investigated a biocomposite graft comprising of chitosan, Nano-hydroxyapatite (nano-HAP), and nano-silver. Instead of adding silver nanoparticles, the study promoted in situ synthesis of nano-silver by utilizing the intrinsic reduction property of

chitosan. The scaffold was highly porous with interconnecting pores in the order of 50–100 μm suited for cell infiltration and growth.

Incorporation of silver in chitosan/nano-HAP biocomposite significantly limits the amount of swelling in the chitosan/nano-HAP scaffolds, which is ideal for implantation and may reduce the amount of stress generated due to the swelling and placed on the surrounding tissues. In vivo persistence of the biocomposite was simulated by incubating the scaffolds in vitro using lysozyme containing buffered medium. The rate of biodegradation can be controlled and reduced through the inclusion of nano-silver. The composite scaffolds were found to be non-toxic to primary calvarial osteoblasts and human osteosarcoma cells. The nano-silver also imparted antibacterial property to the scaffold by significantly inhibiting the growth of *S. aureus* and *E. coli*. Chitosan/nano-HAP scaffold itself possessed antibacterial activity which could be attributed to the presence of chitosan; and upon the inclusion of nano-silver, the activity was increased three-folds. Although the biocomposite was non-toxic, exhibited controlled swelling and biodegradation, and possessed antibacterial activity, its role in biomineralization and differentiation of osteoblasts is yet to be discovered [17].

18.4.2 Chitosan/Nanohydroxyapatite/Nano-Copper-Zinc Composite

Drawbacks associated with the use of chitosan scaffolds such as rapid degradation, poor protein adsorption, non-osteoinductive nature and minimal antibacterial activity were addressed in our research through the additions of nano-HAp and copper-zinc alloy nanoparticles (nano-Cu-Zn) into the chitosan matrix. The scaffolds were fabricated by freeze drying and the produced scaffolds exhibited pores with an average diameter of 115 ± 13.4 μm with high interconnectivity that support cell ingrowth and vascularization. The degradation rates of the chitosan/nano-HAp scaffold with nano-Cu-Zn were significantly reduced compared to the chitosan/nano-HAp scaffolds. Hence, the in vivo persistence of the chitosan/nano-HAp scaffolds would be improved upon nano-Cu-Zn addition.

In order for cellular interaction to take place, the implanted biomaterial should adsorb proteins, which serves as an intermediate for the interaction. The addition of nano-Cu-Zn particles greatly enhanced the protein adsorption onto the scaffolds. We speculated that nano-Cu-Zn alloy particles possessed high surface area and upon its addition, there were more reactive sites for the proteins to be adsorbed. Increased protein adsorbed would ultimately drive more cellular interaction and subsequent biological cascades. As expected, the addition of nano-Cu-Zn alloy nanoparticles improved the antibacterial activity two to three times higher than compared to naive scaffolds. The prepared biocomposite was found to be non-toxic against primary rat calvarial osteoblasts suggesting that it may be a possible and a safer candidate for in vivo applications [18].

18.4.3 Chitosan/Silicon/Zirconium Biocomposite

The additions of inorganic bioactive phases are of great interest in recreating the replica of bone ECM for developing biocomposite scaffolds to support bone regeneration. Silicon dioxide (SiO_2) and silica derivatives have been reported for as bone substitutes and as surface coating to metallic implants to improve bioactivity. The high success rate of implanted biocomposite is dependent upon several parameters and one of the vital parameters is to increase the mechanical strength of the biocomposite. To improve the mechanical properties of chitosan scaffolds, we incorporated zirconia (ZrO_2), as it was able to support osteoblast proliferation, differentiation and no adverse effects are associated with its use. Zirconium (Zr)-based prosthetic devices have been fabricated with high mechanical strength and with no chemical or biological bonding. To improve the osseointegration process, zirconium implants were coated with bioactive glass and HAp. Considering these properties, we developed a chitosan-based biocomposite scaffold containing nanoscale HAp and ZrO_2.

The biocomposite exhibited highly porous interconnectivity, and pore diameters were reduced from 20–60 to 25–40 μm upon nano-Si and nano-Zr addition. The swelling rate was significantly reduced with improvements in the protein adsorption properties. The scaffolds were found to be non-toxic against osteoprogenitor cells. Addition of nano-Si to the scaffolds resulted in increased hydrophilicity which is attributed to the silanol groups (–Si–OH). Increased hydrophilicity will ultimately provide more sites for protein adsorption and thereby enhance the cellular interaction. In vitro biomineralization ability in simulated body fluid (SBF) indicated the ability of the scaffold to deposit hydroxyapatite and osseointegration upon in vivo implantation [64].

18.4.4 Chitosan/Alginate/Nano-SiO_2 Composite

The inclusion of anionic polymers to chitosan-based composites would facilitate electrostatic interaction between the polymeric chains and increase the mechanical strength of the scaffolds. Alginate is an anionic polymer with homopolymeric block of (1-4)-linked β-D-mannurate (M) and its C-5 primer α-L-guluronate (G) residue, respectively by covalent bond. It is biocompatible, hydrophilic and biodegradable under physiological conditions and hence they have been extensively investigated for bone tissue engineering applications. We fabricated a co-polymeric biocomposite scaffold with chitosan and alginate as base polymers, and nano-silica was incorporated into the composite as an inorganic phase.

The scaffold was porous with pore size ranging from 20–100 μm. Without the inclusion of nano-silica, the pore were found to be much larger with a diameter between 20 to 150 μm. Consequently, the addition of nano-silica had a minimal effect on the porosity, but the pores were within the range for allowing cell infiltration and neovascularisation. Interactions between—NH_2 groups of chitosan and—COOH

groups of sodium alginate and Si–OH groups of nano-silica interacted with carbonyl groups of chitosan. These effects significantly reduced pore sizes and swelling ability.

Addition of nano-silica has positive effects on the protein adsorption properties and exogenous biomineralization ability of the scaffolds. Si-O units formed by dissociate Si–OH groups interact with positively charged calcium ions in the solution to form calcium silicate, and afterwards to form amorphous calcium phosphate (ACP) once it interacts with negatively charged phosphate ions. Subsequent stabilization yields nano-crystalline layer. The produced apatite is crucial in recruiting osteoblasts and other cell types to the implant site. Increased surface area and Si–OH presence due to the addition of nano-silica is found to have improved the protein adsorption ability of the scaffold.

Scaffolds containing nano-silica exhibited a compressive stress and Young's modulus of 0.59 ± 0.405 MPa and 8.16 ± 0.567 MPa, respectively. On the other hand, chitosan/alginate scaffolds recorded a compressive stress and Young's modulus of 0.66 ± 0.02 MPa and 8.99 ± 0.016 MPa, respectively. There was nominal increase in the compressive stress, but there was no effect on the Young's modulus. The scaffolds were relatively safe to mammalian cells as they exhibited no discrete cytotoxicity to rat osteoprogenitor cells, and human osteoblastic cells [65].

18.4.5 Chitosan/Diopside Scaffolds

The addition of bioceramics to chitosan-based scaffolds has shown to improve both physicochemical and biological properties. As a bioceramic, diopside which supports osteoblast cells attachment, proliferation, differentiation and induce apatite formation, has been extensively investigated. Using diopside by itself as an osteogenic element would not be ideal in the treatment of bone defects with various geometries. In addition, the dissolution rate of diopside would be uncontrollable. Hence, we utilized chitosan scaffolds to support the addition of diopside and examined its potential to augment bone formation. Diopside particles were synthesized from rice straw ash (RSA), and its addition to chitosan matrix produced a porous biocomposite scaffold with high crystallinity. Diopside particles interacted through its Si–OH groups with—NH_2 groups of chitosan, thus the swelling of naive chitosan scaffolds is controlled. Although the addition of diopside particles decreased the swelling properties of the scaffold, it had no influence on the rate of biodegradation in the presence of lysozyme. Hydrated scaffolds also exhibited protein adsorption ability to support subsequent cellular interaction. Silanol groups of diopside and carboxyl, hydroxyl groups of chitosan are the primary functional groups responsible for protein adsorption.

As observed in one of our earlier study, the incorporation of nano-silica promoted biomineralization due to the presence of Si–OH groups, similarly, the inclusion of diopside particles also improved the rate of biomineralization upon its exposure to SBF. The biocomposite scaffolds were compatible to human osteoblastic cells with no discrete cytotoxicity in vitro. Ascertaining the role of the composite scaffold in

regulating osteoblast activity, we discovered that alkaline phosphatase (ALP) and type 1 collagen (Col-1) mRNA expression were increased under osteogenic supplements concluding the osteoconductive nature of the prepared scaffolds. Upon in vivo implantation in a rat thigh muscular pouch model, decreased neutrophils infiltration and increased collagen deposition was clearly observed within the biocomposite scaffolds. Based on these observations, it was concluded that the scaffolds were highly biocompatible in vivo and suitable for bone tissue engineering applications [66].

18.4.6 Chitosan/Carboxymethyl Cellulose/Mesoporous Wollastonite Particles Scaffolds

Carboxymethyl cellulose (CMC) is an anionic polymer which has the strong ability to establish an electrostatic interaction with the cationic polymer chitosan. Crosslinking of chitosan with CMC was found to enhance the swelling and protein adsorption properties. To increase the bioactivity, ceramic particles such as bioactive glass have been incorporated and utilized for applications in bone tissue engineering. In this respect; we added mesoporous wollastonite particles to chitosan/CMC matrix. Our research group previously reported that mesoporous wollastonite synthesized from rice straw ash had osteogenic potential with the ability to bridge critical-sized tibial defects in rat models. Hence, we combined mesoporous wollastonite along with chitosan/CMC scaffold to fabricate a functional scaffold with inorganic phase similar to bone tissue.

Tailored addition of mesoporous wollastonite particles with different concentration from 0.25–1% was investigated. Chitosan/CMC scaffolds with and without mesoporous wollastonite particles exhibited pores with 100 μm diameter and no change in the pore dimensions were observed at different concentrations (Fig. 18.2).

The addition of mesoporous wollastonite particles regardless of concentration will result in the chitosan/CMC scaffolds to exhibit exogenous biomineralization ability in simulated body fluid (SBF) with HAp deposition on the surface and inside the pore walls.

Upon hydration, the presence of silanol group in the mesoporous wollastonite particles tends to improve protein adsorption. In comparison to other concentrations, protein adsorption was found to be the highest with the addition of 0.5% mesoporous wollastonite particles. The swelling was controlled upon mesoporous wollastonite addition to chitosan/CMC matrix which would enhance the mechanical properties stability in vivo. The scaffold was found to be non-toxic to human osteoblasts and supported cell growth with no alteration in the metabolic activity.

The biocomposite promoted MSCs differentiation into osteoblasts in the presence of osteogenic stimulants. Mouse mesenchymal stem cells were osteodifferentiated when incubated for 9 days in conditioned medium obtained from the scaffolds. As identified by alizarin red staining, calcium deposits in response to the biocomposite

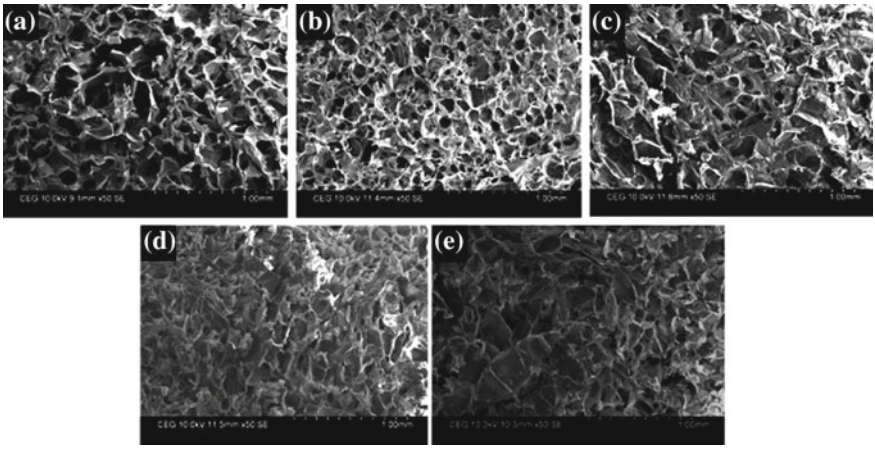

Fig. 18.2 Scanning electron microscopic images of the scaffolds. **a** Chitosan/CMC scaffolds. **b–e** correspond to chitosan/CMC with 0.25, 0.5, 0.75 and 1% mesoporous wollastonite particles, respectively. Pores greater than 100 μm in size were observed. (Reprinted with permission from [67], Elsevier publishing group)

scaffold were higher under osteogenic induction proving the osteoconductive nature of our scaffold.

The interaction between biomaterials and cells leads to the activation of various intracellular cascades that regulate cellular processes of proliferation, survival, and differentiation. Micro-RNA's (miRNA) are small non-coding RNA's spanning 19–25 nucleotides in length is found to act as post transcriptional regulators. Induction of specific miRNAs is found to determine the fate of cells in contact with biomaterials. Hence, in this study, we identified pre-miR-15b, an osteoblast specific miRNA to be upregulated in mouse mesenchymal stem cells (mMSCs) by the chitosan/CMC/mesoporous wollastonite scaffolds. Hence, the connection between biomaterial composites and cells was delineated at molecular level [67].

18.4.7 Chitosan/Graphene Oxide Scaffolds

Carbon based materials are appealing and their inclusions in the form of carbon nanotubes, fullerene, graphene oxide, carbon nanofibers are widely studied to increase hydrophilicity, impart electrical properties, and osteoinduction. Graphene oxide supports cell proliferation and osteogenic differentiation by modulating the cytoskeletal arrangement and cytoskeletal tension. Addition of graphene oxide to chitosan has improved the mechanical properties of several folds and improved osteogenic properties [59].

Our team fabricated chitosan scaffolds containing 0.1−1% graphene oxide. The addition of graphene oxide significantly improved the hydration properties due to the

hydrophilicity imparted by graphene oxide sheets. The addition of 0.25% graphene oxide to chitosan displayed an increase in the amounts of proteins that can be adsorbed on the scaffolds. In addition, the inclusion of graphene oxide also reduces the amount of degradation of the scaffold and it was observed only 40% of the scaffolds were lost at the end 30-day incubation period in the presence of lysozyme. The inclusion of graphene oxide also significantly increased the biomineralization potential of chitosan scaffolds. The scaffolds were discovered to be non-toxic to mesenchymal stem cells (MSCs) and promoted its osteogenic differentiation by upregulating ALP, Runt related transcription factor −2 (Runx2), Col-1 and osteocalcin marker genes. Runx2 protein levels were increased in response to the scaffolds under osteogenic stimulants concluding the osteoconductive nature of the scaffolds. In vivo implantation of the scaffolds in critical-sized rat tibial bone defects (Fig. 18.3) significantly healed the defects and promoted wound closure with new bone formation and ECM deposition after two weeks post-implantation [59].

18.4.8 Chitosan-Based Biocomposite Scaffolds

Chitosan-based biocomposite scaffolds were fabricated with the additions of various copolymers, ceramic particles and nanoparticles to improve protein adsorption prop-

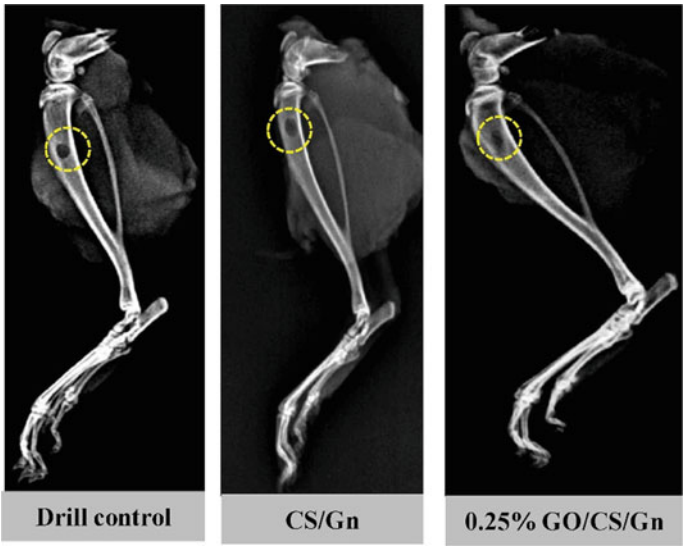

Fig. 18.3 Healing of rat tibial bone defects in vivo. Representative X-ray photographs of the rat tibial bone defects of control clot (no filler), filled with chitosan/gelatin scaffolds or 0.25% graphene oxide/chitosan/gelatin scaffolds after two-week post-implantation. (Reprinted with permission from [59], Elsevier publishing group)

erty, control swelling and degradation rate, enhance biomineralization and modulate cellular response. A brief literature survey on other biocomposites based on chitosan is represented in Tables 18.1 and 18.2.

18.4.9 Thermo-Responsive Chitosan Hydrogels for Bone Repair

Chitosan-based materials are useful in bone tissue engineering in the form of hydrogels. Hydrogels are hydrophilic polymeric networks which can absorb several folds of water without disintegrating its structure. Prefabricated scaffolds requires repeated surgical procedures to implant the graft whereas, hydrogels can be injected into the site of defect and can adapt defects of any geometry with a minimally invasive approach [104]. Chitosan-based hydrogels are widely investigated in bone tissue engineering include chitosan, chitosan/glycerophosphate, chitosan/lactide, chitosan/poly(ethylene oxide) and chitosan-g-poly(N-isopropylacrylamide).

Hydrogels can confront complex geometries upon implantation and can be crosslinked by chemical crosslinkers, adjustment in temperature, pH, and photocrosslinking. They can carry cells and biomolecules in the original form due to the presence of large amounts of water. Hydrogels are remarkable candidates to be used in sites that require minimal mechanical strength or in non-load bearing sites [105, 106]. Amongst the group of hydrogels, thermosensitive injectable hydrogels possess various advantages include minimal invasiveness, ability to carry therapeutic bioactive molecules, cells, and drugs. Polymers' responding to temperature is associated with the ability to exert negligible adverse reactions to the living tissues compared to other responsive systems such as electric field or pH based approach [104].

Chitosan lacks thermosensitivity, and hence, thermal gelation is often achieved by the addition of polyol salt such as glycerophosphate to the system. Chitosan is highly insoluble in neutral or basic pH while soluble in acidic conditions with a pKa of 6.5. The solubility of chitosan occurs from the protonation of free amino groups in the backbone of the chitosan structure. Neutralization to pH beyond 6.2 exhibits a hydrate gel-like precipitate formation with no gel formation. Phase separation of chitosan is possible only with pH >6.0 to form a complete hydrogel. Therefore, maintaining chitosan solutions below room temperature and addition of glycerophosphate solution forms a gel within the physiological pH range. The solution remains as liquid at room temperature and solidifies into gel when the temperature is raised to 37 °C [47].

The addition of glycerophosphate salt to the chitosan system controls nearly all interactions include electrostatic, hydrogen bonding and hydrophobic interactions. Glycerophosphate inclusion plays three crucial roles: (1) increases the pH from acidic range to acceptable physiological limit (7.0–7.4); (2) provides thermogelling property at 37 °C; and (3) inhibits immediate precipitation of the CS solution [107].

The in situ forming of chitosan hydrogels was reported initially by Chenite et al. [107] and it was discovered that the hydrogel was able to encapsulate chondrocytes.

Table 18.1 Materials used in the synthesis of chitosan-based biocomposites and the properties observed (Reprinted with permission from [21], Elsevier publishing group)

Chitosan+Polymeric+Ceramic Biocomposite	Properties observed	References
Alginate and Chondroitin 4-sulfate	• Increased compressive modulus and enhanced apatite formation; • Promoted cell spreading, proliferation and osteogenic differentiation of bone marrow derived stem cells (BMSC) by increasing ALP activity	[68]
Alginate and HAp	• Controlled and uniform porosity; • Increased compressive strength and elastic modulus; • Promoted differentiation and mineralization of MC3T3-E1 cells	[69]
Alginate and In situ synthesized HAp	• Highly interconnected porosity with thicker pore walls	[70]
Alginate and Nano-SiO_2	• Improved protein adsorption, controlled swelling; • Improved apatite deposition	[65]
Alginate, Collagen, and HAp	• Exhibited higher proliferation and mineralization of cells	[71]
Alginate and Polypyrrole	• Controlled swelling by decreasing the porosity and lowered biodegradation; • Improved cell viability and cell attachment; • Biomineralization in SBF and under culture conditions were enhanced	[72]
Carboxymethyl cellulose and mesoporous wollastonite	• Reduced swelling; • Decreased susceptibility to lysosome; • Increased biomineralization; • Upregulated miRNA pre-mir-15b	[67]
Chitin and Nano ZrO_2	• Controlled swelling and degradation; • Enhanced osteogenesis	[31]

(continued)

Table 18.1 (continued)

Chitosan+Polymeric+Ceramic Biocomposite	Properties observed	References
Chondroitin Sulphate, Amylopectin, and HAp	• Increased the Cell proliferation, ALP activity and collagen-1 expression in MG-63 cells	[73]
Collagen, Vascular endothelial growth factor (VEGF) loaded PLGA-polyethylene glycol (PEG) microspheres, and human adipose derived stem cells (ASCs)	• Promoted vascularisation in vivo in rats	[74]
Fucoidan and β-TCP	• Improves the compression strength; • Enhanced apatite deposition; • Increased osteocalcin secretion by hMSCs	[75]
Gelatin and Calcium phosphate	• Increased material degradation and efficient resorbability; • Improved cell attachment	[76]
Gelatin, HAp, and Montmorillonite	• Decreased degradation rate; • Increased apatite deposition and swelling property	[77]
Gelatin and Nano-SiO_2	• Increased the swelling ability; • Decreased degradation rate; • Improved protein adsorption and biomineralization; • Enhanced cell attachment and ALP activity	[78]
Gelatin and β-TCP	• Improved compressive strength by 70%; • Increased the swelling and biomineralization	[79]
Keratin nanoparticles	• Increased protein adsorption and improved biodegradation	[80]
Mimosa tenuiflora cortex	• Improved cell viability, proliferation and differentiation and ALP expression in rat cavarial cells	[81]

(continued)

Table 18.1 (continued)

Chitosan+Polymeric+Ceramic Biocomposite	Properties observed	References
Poly-3-hydroxybutyrate-co-3-hydroxyvalerate (PHBV) and HAp	• High ultimate tensile strength upon varying HAp addition; • Significantly higher ALP activity in human fetal osteoblasts; • Chelated apatite crystals and increased biomineralization	[82]
Polycaprolactone	• Improved bioactivity, protein adsorption and adhesion of osteoblasts	[83]
Poly(l-lactic acid)	• Tunable porosities	[84]
Poly-l-glycolic acid (PLGA) nanoparticles and bioactive glass	• Increased the mechanical strength; • Reduced swelling behaviour	[85]
PLGA nanocapsules loaded with BMP-2 and PHBV loaded with BMP-7	• Supported MSC attachment and spreading; • Sequential delivery of growth factors; • Enhanced differentiation and increased ALP activity	[86]
PLGA microparticles loaded with simvastatin	• Reduced swelling; • Increased compressive modulus; • Enhanced cell proliferation of hFOB and improved differentiation	[87]

(continued)

Table 18.1 (continued)

Chitosan+Polymeric+Ceramic Biocomposite	Properties observed	References
Poly(lactic acid-glycolic acid) microspheres	• Porosity was controllable; • Matched the compressive modulus and strength of trabecular bone; • Increased ALP activity; • ALP, osteopontin (OPN), bone sialoprotein (BSP), and OCN levels were upregulated	[88]
Poly(propylene carbonate)	• Enhanced hydrophilicity favouring fibroblast attachment and proliferation; • 26% increase in Young's modulus	[89]
Poly(vinyl alcohol), Collagen, and Bioglass particles	• Controlled porosity, swelling and degradation; • High compressive modulus; • ALP activity was increased; • Controlled release of protein (BSA)	[90]
Polyvinyl pyrroidone and 45S5 Bioglass	• Genipin crosslinked decreases degradation; • Supported cell growth by controlling the dissolution of bioglass; • Improved cell attachment with extended cytoplasmic processes with polygonal morphology	[91]
Silk Fibroin	• Decreased the degradation rate and possessed antibacterial activity; • Supported adhesion and growth of fibroblasts	[92]
Silk Fibroin and HAp	• Reduced the porosity; • Supported growth of SaOs-2 cells and increased viability; • Enhanced ALP activity	[93]
Silk Fibroin and Nano ZrO_2	• Increased water uptake ability and compressive strength by controlling the porosity	[94]
β-1,3-glucan and HAp	• Favoured cell adhesion, spreading and proliferation and upregulated the ALP, OC, Col-I levels	[95]

Table 18.2 Biological, bioceramics, and nanomaterials used in the synthesis of chitosan-based biocomposites and the properties observed (Reprinted with permission from [21], Elsevier publishing group)

Chitosan+Biological+Bioceramics +Nanomaterials	Properties observed	References
Bioactive glass	• Increased compressive modulus	[96]
Bioactive glass nanoparticles	• Exhibited shape memory properties; • Induced apatite formation	[97]
Bioglass	• Controlled pore morphology upon varying the amount of Bioglass; • Better release kinetics of gentamicin sulfate	[98]
Bioglass and carbon nanotube (CNT)	• Increased mechanical properties and compressive strength; • Promoted attachment and proliferation of MG-63 cells	[99]
CNT (Multi-walled)	• Promoted water uptake ability; • Controlled the porosity; • Enhanced cell proliferation, protein content and ALP activity; • Mineralization was higher	[58]
HAp and β-tricalcium phosphate (β-TCP)	• Enhanced tensile properties; • Controlled water retention and maintained structural stability	[100]
HAp and Iron oxide nanoparticles	• Promoted bone healing upon magnetic stimulation	[101]
β-TCP	• TCP addition decreased the degradation of scaffolds	[102]
RGD	• Increased pore size and improved mechanical properties; • Cell attachment and proliferation was promoted; • Increased calcium deposition	[103]

The hydrogel was biocompatible, and upon subcutaneous injection into athymic mice, the encapsulated cells secreted ECM similar to cartilage tissue. Hydrogel should exhibit relatively less gelation time with good pH stability for bone tissue engineering applications. Tailoring the addition of β-glycerophosphate influences the gelation time, pH stability and cytotoxicity. Increased addition of gelling agents will ultimately result in hypertonicity and eventually cause death to the encapsulated and surrounding cells. On the other hand, reduced gelling agents leads to loosened structures and faster degradation. Adjustment in the concentration of gelling agents should, therefore, be considered seriously in designing a biocompatible hydrogel with optimal gelation time and mechanical properties.

Although chitosan-glycerophosphate hydrogels are flexible and it can orient into any geometrical defects, it is associated with less mechanical strength and poor

osteoinduction properties. A combinatorial approach is needed to negate various deleterious conditions associated with bone defects such as hypoxia, decreased osteoconduction, microbial infections and poor vasculature [3, 6]. Hence, hydrogel satisfying the aforementioned properties would be an ideal candidate for bone tissue engineering. In this aspect, glycerophosphate containing CS-based hydrogels have been modified with the addition of various bioactive elements, polymers and secondary carriers for drug release.

18.4.10 Zinc-Chitosan-Glycerophosphate-Nano-HAp Hydrogels

To impart antibacterial properties to the chitosan hydrogel, zinc was doped into chitosan by its intrinsic reduction property, and glycerophosphate based hydrogel was fabricated. Zinc is found to inhibit osteoclast differentiation, promotes osteoblastic activity and aid bone formation [108]. The hydrogel gelled at staggering 5 min incubation period at 37 °C and possessed controlled swelling ability. The hydrogel was found to be non-toxic to human osteoblastic cells and promoted osteoblast differentiation under osteogenic conditions by promoting bone mineral deposition. Presence of zinc in the hydrogel imparted superior antibacterial properties compared to naive chitosan hydrogel [109].

The addition of HAp, a mineral component of natural bone tissue, to a polymeric matrix will provide osteoinductive and osteoconductive properties. Its addition is associated with improved bone bonding abilities and reduced degradation in situ. Furthermore, the nanoscale dimensions of HAp enhance the protein adsorption, cell interaction, and better osteogenic properties. Nano-HAp was included in zinc-chitosan/glycerophosphate hydrogel system to assess the improvement in biological properties in vitro and in vivo [109, 110].

The addition of nano-HAp into zinc-chitosan/glycerophosphate hydrogel improved swelling ability by increasing the hydration property and therefore enhanced protein adsorption on to the hydrogel. Exogenous biomineralization with deposition of HAp crystals was promoted upon nano-HAp addition. Interestingly, there was no difference in the degradation rate and antibacterial activity was observed in hydrogels with and without the addition of nano-HAp.

From an in vitro perspective, the hydrogels promoted mesenchymal differentiation towards osteogenic lineage by upregulating various osteoblast related markers include Runx2, COL-1, ALP, and osteocalcin. At cellular level, the composite hydrogel promoted mineralization of the MSCs with increased calcium nodules observed under osteogenic stimulants. The prepared hydrogel was concluded to be osteoconductive in nature. During in vivo observations, the composite hydrogel significantly promoted healing and bridging of rat tibial bone defects. Hence, based on the above observations, the zinc-chitosan/nano-HAp/glycerophosphate hydrogel is found to be suitable for in vivo applications in treating bone defects [110].

18.5 Conclusions

This chapter reviewed and discussed several chitosan-based biocomposite scaffolds and hydrogels that were fabricated and investigated by our research group. The ideal scaffolding properties are discussed to facilitate the readers for designing scaffolds based on chitosan to meet their applications. Chitosan itself possess various properties suited for bone tissue engineering but lacks certain vital properties. Hence, addition of copolymers and other nanoparticles to fabricate composites have greatly improved its properties and accounted for the missing functions to support bone formation. Tissue engineers around the globe choosing chitosan as base polymer for developing functional bone grafts may consider the inclusion of cells along with copolymers, bioactive molecules, and nanoparticles to further enhance the cell-material interaction to quicken bone regeneration.

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Chapter 19
Marine Polysaccharides: Biomedical and Tissue Engineering Applications

Shashiaknt Joshi, Shruthi Eshwar and Vipin Jain

Abstract Natural polysaccharides of marine origin are gaining interest in biomedical applications. Seaweeds are most abundant source of polysaccharides, as alginates, agar and agarose as well as Carrageenans. Even cellulose and amylose have been extracted from the macroalgae. Chitin and chitosan are derived from the exoskeleton of marine crustaceans. Interdisciplinary fields involving various science and technology aspects such as cell sciences, biomaterials, medical sciences and engineering are referred to as tissue engineering, which is an upcoming new field intended to replace biological functions in human body. Tissue engineered scaffolds and artificial organs developed by such technique has replace injured parts in human body. Technological advancements have made it possible to obtain active ingredient in marine organisms by controlling the growth and isolation conditions. Present review has focused on progress in discovering and producing new applications of marine polysaccharides in biomedical and tissue engineering.

Keywords Marine polysaccharides · Tissue engineering · Natural biopolymers · Fucidane · Carrageenan · Ulvan · Chitin · Chitosan

19.1 Introduction

Polysaccharides are linear/branched polymer molecules comprising of alternating units of sugar connected by glycosidic bond. Polysaccharides function as structural components and energy tank in biological systems. Polysaccharides such as agar, cellulose, alginate, xylan, carrageenan and chitin are considered as insoluble complex polysaccharides. In water sources insoluble complex polysaccharides are enormous

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in variety such as seaweeds, fungi, crustaceans etc., which may be soluble or insoluble in water or present in semi crystalline form. Marine microorganisms have major role in nutrient recycling.

Aquatic sources have enormous biomaterials for human mankind. Three-quarters of the planet has water in the form of oceans, rivers and lakes amongst which 97% of water is occupied by in the oceans with vast marine resources like bacteria, algae, fungi and fishes which exhibit defensive functions and allows the organisms to survive in that environment. The biomaterials derived from marine sources are an innovative area of research with significant claim in biomedical field. As a result of vast diversity and biological properties, marine derived bio-silica, polysaccharides, enzymes, lipids, proteins, toxins and algae-based products have wide applications in biological and biomedical fields. During this present decade, marine biomaterials have attracted the attention of the researchers and industry to develop products commercially. Biomaterials include metallics, ceramics, synthetic polymers and natural biopolymers for variety of biomedical and tissue engineering applications.

Marine biomaterials have significant potential, complete research is mandate to study the development of products with certain goals like:

1. Discovery of materials to understand their action and functions.
2. Study the understanding of physical, mechanical and biological properties.
3. Develop associated instrumentation or sensors which can aid in diagnosis.
4. Marine biopolymers or their derivatives and its application in biomedical field.

19.1.1 Materials Used in Tissue Engineering

Marine biomaterials include seaweeds, crustaceans and algae, which include various polymeric substances ranging from mono-saccharides to polysaccharides like chitin, chitosan, carrageenan, fucoidan, alginate and ulvan. These biopolymers after extraction, purification have significant role in biological and biomedical field, such as dental, wound healing, ophthalmology, orthopaedics, tissue engineering applications:

1. Biopolymers, bioactive glasses or bio-silica as ceramics material mainly phosphates or sulphate salts;
2. In composites with synthetic polymers have shown to increase strength of scaffold: polyfumarates, polylactic acid (PLA), polyglycolic acid (PGA), polycaprolactone, Copolymers of PLA and PGA.

Research in tissue engineering has gained significant interest in biomaterials and interdisciplinary approaches. They mimic extracellular matrix, initiate the cellular responses and guide tissue formation which are important features of regeneration.

19.1.2 *Overview of Tissue Engineering and Related Applications*

Interdisciplinary scientific approach has been taken to develop wide range of medical devices as shown in Fig. 19.1.

Tissue engineering is an interdisciplinary research combining cell engineering, materials methods and suitable biochemical/physicochemical factors to improve or replace biological function. Engineering design by using computer aided modulation and simulation is combined with bio-materials structures to produced new product. Extensive evaluation of prototypes are done before it is used to treat defective organ in human body [1]. The term is created and applied to perform specific biochemical functions using cells within an artificially created support system. Multiple approaches are possible once engineering sets in; however, four methodologies are prominently used for tissue engineering. The first approach includes the application of biomaterials as scaffolds, which are structures prepared to create an artificial cellular environment [2]. The second approach is utilization of stem cells for creating artificial tissues and organs either with or without polymers [3, 4, 50]. The third approach includes use different biomaterials to construct scaffolds and bio signals [5], which are substances normally present in tissues and capable of stimulating cellular growth, proliferation and cellular differentiation. Finally, the fourth approach includes scaffold, bio signals and cells together in order to minimize the differences between the artificial and the cellular environment; the three key elements-cells, scaffolds and signals develop a sequence of events to initiate regeneration [6]. The three-dimensional biodegradable materials or scaffolds support tissues firmly bind with host tissues and serve as temporary space for tissue regeneration.

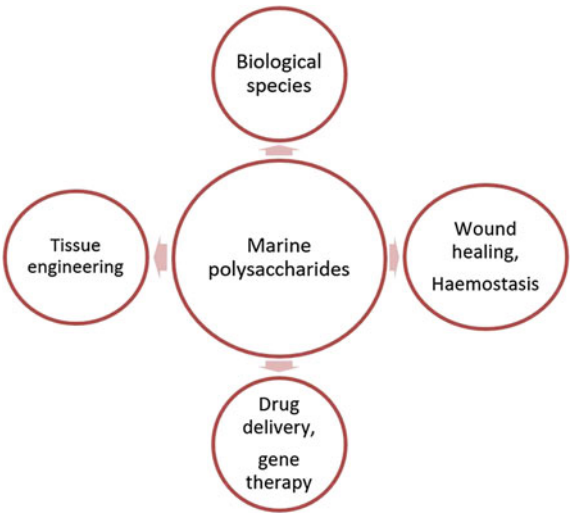

Fig. 19.1 Overview of marine polysaccharide applications

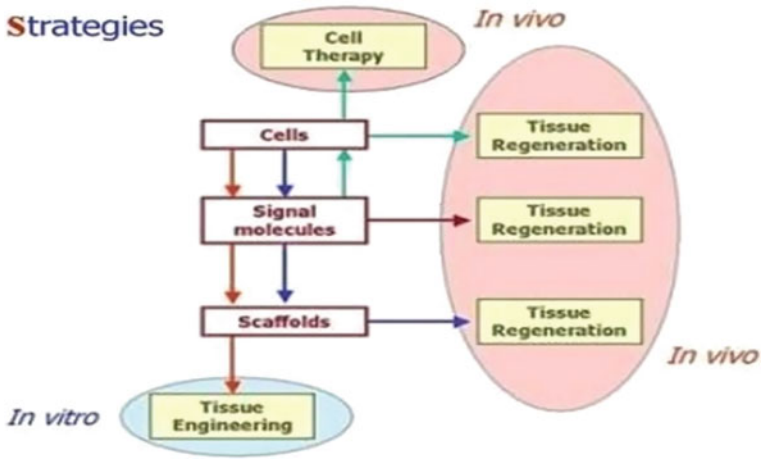

Fig. 19.2 Schematic approach for tissue regeneration

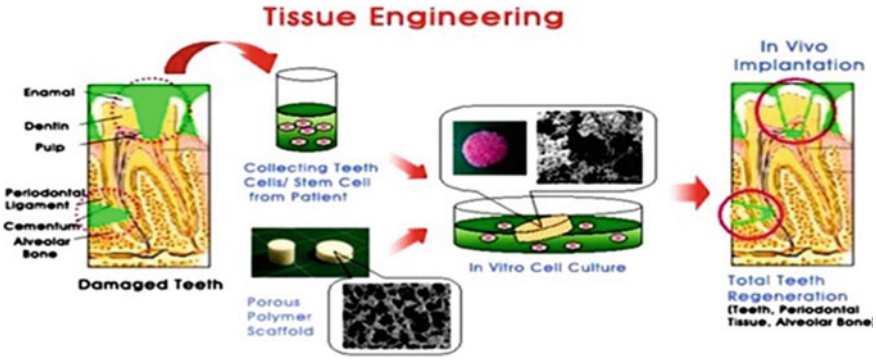

Fig. 19.3 Process flowchart for dental application

A number of techniques are available in the production of scaffolds: nanofiber self-assembly; textile technology; solvent casting; gas foaming; emulsification; freeze drying; thermally induced phase separation; electro-spinning; CAD/CAM design simulation; and laser assisted bio-printing. Marine organisms are ideal starting materials for bone, cartilage and skin differentiation as they are porous, biocompatible, biodegradable and exhibit osteo-inductive and osteo-conductive properties of bone.

Wide range of pharmaceutical uses have been developed with marine polysaccharides. Various methodologies used are shown in Fig. 19.2.

For dental application one can use following procedure (Fig. 19.3).

Marine polysaccharides are used for wide range of pharmacological applications as shown in Fig. 19.4.

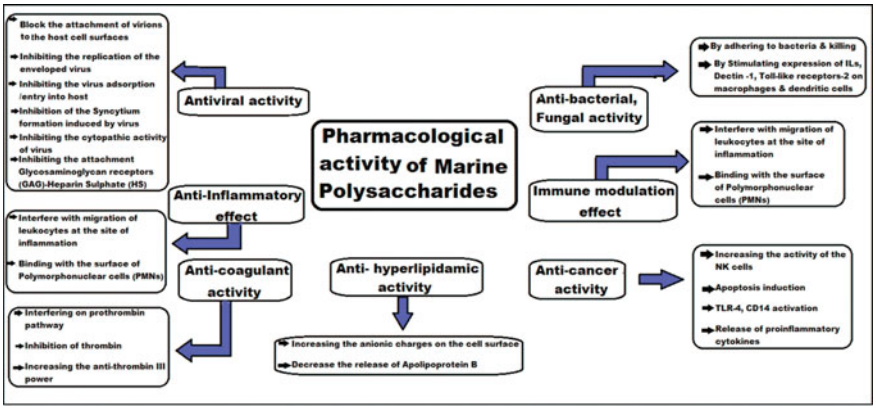

Fig. 19.4 Wide range of pharmacological applications using marine polysaccharides

19.2 Algal Polysaccharides—Introduction and Its Importance

Algal polysaccharides are used widely in biomedical field as they are functionally active, biodegradable and water soluble. Marine algae are a rich source of compounds constituting to ~15% of ocean's storage. Research has proven that algal polysaccharides are superior bioactive polymers exhibiting various functions like anticoagulant, immune mediator, antioxidant, anti-tumour, anti-inflammatory and antimicrobial [7].

Marine algae are polymers with simple sugar units linked by glycosidic bonds. They contain good amounts of cell wall polysaccharides, myco-polysaccharides and storage polysaccharides. Amongst algal polysaccharides seaweeds are present in range of 5–76% with highest concentrations found in green seaweeds.

Current scenario finds rapid advancement in polysaccharide biotechnology and potential biological properties offer extensive opportunity in biomedicine particularly in tissue engineering and drug delivery.

These are classified as brown, red and green seaweeds as shown in Fig. 19.5.

19.2.1 Alginate from Brown Algae

Alginate is an anionic polysaccharide present along the cell wall of seaweeds includes 45% of algae along with other proteins and polysaccharides. The source of alginate is mainly from Laminaria Macrocystis, Ascophyllum, Eclonia, Lessonia, Durvillea and Sargassan species. Alginate comprises of β-D Mannuronic acid (M) and α-L guluronic acid (G) linked by 1-4 glycosidic bonds. The (M) and (G) are stereo-isomers which occur individually as M/ G or in mixed sequence (MG). The occurrence influences the properties. Alginate is known for its gelling capacity which is due to

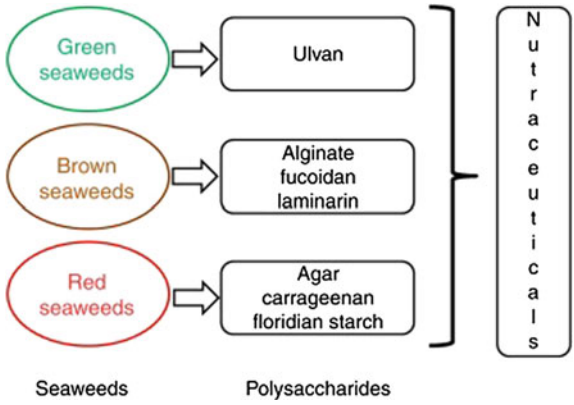

Fig. 19.5 Marine origin materials and isolated polysaccharides

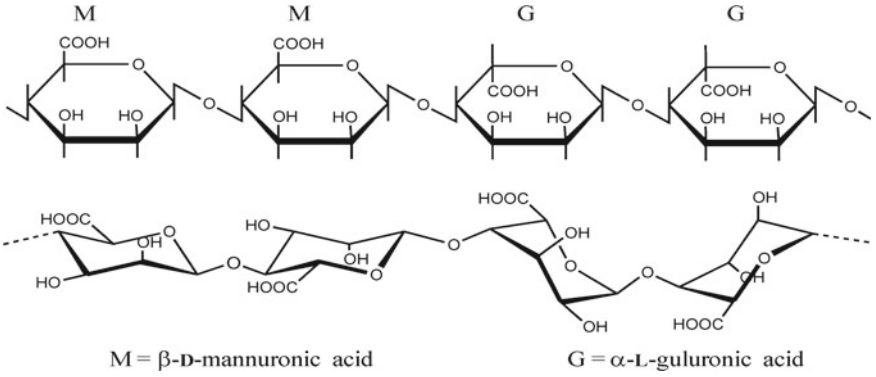

Fig. 19.6 Polymeric structure of alginate

the presence of gelling agent, its structure and molecular weight. The presence of G monomers helps in gelation by forming ionic bridges. Alginate rich in G monomer will be stiffer and more brittle, whereas alginate rich in M monomers is more flexible.

19.2.1.1 Structure and Isolation

See Figs. 19.6, 19.7 and 19.8.

19.2.1.2 Applications

Characteristics like low toxicity, good biocompatibility, inexpensive and capability to form hydrogel at lower pH have gained broader usage of the polymer in drug delivery, tissue engineering, cell mobilization and wound healing.

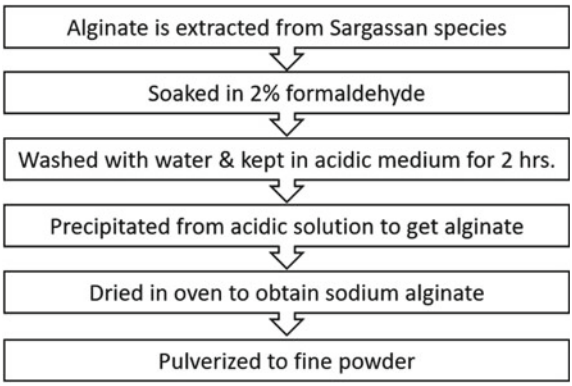

Fig. 19.7 Extraction and purification of alginate

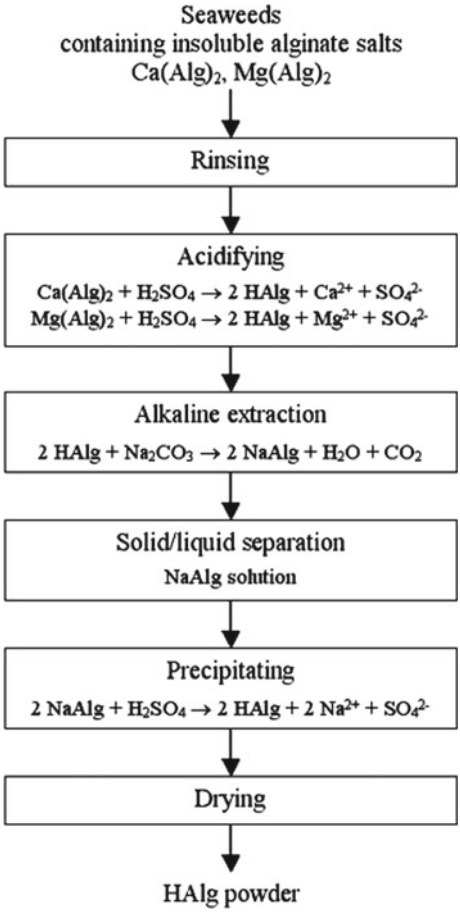

Fig. 19.8 Seaweeds extraction process for alginate production

Tissue Engineering

The strength of the matrix or scaffold has been proven to be enhanced when alginate is incorporated within the biocomposite. Alginate hydrogel along with stem cells, growth factors have shown to achieve osteogenic potential with increased AL-phase activity, osteocalcin and mineralization expression. Alginate gels are known to cause cartilage and bone regeneration. Bone tissue engineering applications include regeneration of bone after tissue loss due to degenerative, surgical, or traumatic processes, as well as applications such as spinal arthrodesis. In addition, there is the desire to speed the healing of bone fractures and to treat established nonunion fractures [8].

Recent Studies on Alginate in Bone Tissue Engineering

The alginate/hydroxyapatite composite scaffolds were prepared by internal gelation followed by a freeze-drying procedure to obtain a porous structure. The nanoparticles were prepared in presence of a lactose-modified chitosan, and this colloidal solution was adsorbed on the scaffolds by exploiting electrostatic interactions. They are used as temporary restorable bone implants [9]. Chitosan/polypyrrole/alginate composite scaffold can act as a substrate for tissue regeneration and this can be employed for bone tissue engineering using osteogenic cells by utilizing electrical stimulation with a bioreactor system and thereby evaluate the role of conducting substrate in bone regeneration [10]. Calcium phosphate cement (CPC) paste combined with AC (alginate-chitosan) microcapsules encapsulating MC3T3-E1 cells were studied in vivo by Qiao et al. [11]. Alginate-chitosan microcapsules are better than alginate ones for seeding cells deep into injectable CPCs and the novel injectable CPCAC-cell construct is promising for bone tissue engineering applications [12]. The preparation of novel porous scaffolds of CPC combined with alginate is used as a three-dimensional (3D) matrix for drug delivery and tissue engineering of bone [13]. Alginate incorporated scaffold with 3D-plotted mesoporous bioactive glass (MBG) has well-ordered nano-pores as well as controllable large pores. This scaffold has significantly improved physicochemical, biological, and drug-delivery properties that can be a good platform for bone tissue engineering [14].

Drug Delivery

Alginate is being used as binder, gelling agent, stabilizing agents in tablets [15]. Furthermore, they are combined with multiple drugs for delivery [16]. Alginate can provide immediate and sustained drug release [17]. Lastly, alginate matrix is used for delivery of proteins and DNA [18].

Greater emphasis is placed on biopolymer-based hydrogels for their potential use as carriers in controlled drug delivery. Recently, much research efforts have been concentrated to develop calcium alginate beads loaded with various low molecular weight therapeutic agents. In a number of studies, alginate beads have been used as excellent vehicles. Polymethyl-methacrylate (PMMA) coated microcapsules of diclofenac

sodium (DFS) were prepared by a modified water-in-oil-in-water (W1/O/W2) emulsion solvent evaporation method using sodium alginate as a matrix material in the internal aqueous phase. Their performance with respect to controlled release of the drug in simulated gastric fluid and simulated intestinal fluid were evaluated and compared with non-matrix microcapsules prepared by the conventional W1/O/W2 emulsion solvent evaporation method. The matrix microcapsules appeared to be suitable for releasing lesser amounts of DFS in simulated gastric fluid and providing extended release in simulated intestinal fluid [19]. Sustained release microparticles were successfully prepared by Raja Chakraverty [20] by employing ionotropic gelation technique where the natural water-soluble polymer, namely, sodium alginate prolongs the release of the drug Norfloxacin. Chitosan coated alginate microcapsules were developed as oral sustained delivery carriers for anti-tubercular drugs in order to improve patient compliance, to reduce dose/dosing frequency in the management of tuberculosis (TB), which otherwise demands prolonged chemotherapy. The microcapsules exhibited a slow and sustained release over a period of 72 h.

Wound Healing

Calcium in alginate undergoes ionic exchange forming a gel which amplifies clotting mechanism and brings haemostasis [21].

Alginate-based wound dressings offer many advantageous features. Traditional wound dressings (e.g., gauze) have provided mainly a barrier function—keeping the wound dry by allowing evaporation of wound exudates while preventing entry of pathogen into the wound [22]. In contrast, modern dressings (e.g., alginate dressings) provide a moist wound till tissue build up [47].

Blends of alginate, chitin/chitosan, and fucoidan gels have been reported to provide a moist healing environment in rats, with an ease of application and removal [23]. The addition of silver into alginate dressings also enhanced the antioxidant capacity [24]. Alginate fibres cross-linked with zinc ions have also been proposed for wound dressings, as zinc ions may generate immune-modulator and anti-microbial effects, as well as enhanced keratinocyte migration and increased levels of endogenous growth factors [25].

Nerve Repair

Alginate gels have also been investigated for the repair of the central and peripheral nerve systems. Alginate-based highly anisotropic capillary gels, introduced into acute cervical spinal cord lesions in adult rats, were integrated into the spinal cord parenchyma without major inflammatory responses and directed axonal re-growth [26].

19.2.1.3 Advantages and Limitations

The advantages of alginate gels in bone and cartilage regeneration include [27]:

1. Ability to be introduced into body in a minimally invasive manner;
2. Ability to fill irregularly shaped defects;
3. Ease of chemical modification with adhesion ligands;
4. Controlled release of tissue induction factors such as bone morphogenetic protein (BMP), TGF-β (transforming growth factor beta); and
5. Both drugs and cells can be readily integrated into the scaffolding matrix.

However, the disadvantage of using alginate gels include:

1. Insufficient mechanical properties to allow load bearing in initial stages of regeneration without fixation; and
2. Currently, alginate is still unable to meet all the design parameters simultaneously (e.g., degradation, bioactivities or mechanical properties).

19.2.2 Fucoidan from Brown Algae

Fucoidan is a sulphated hetero-polysaccharide present along the cell wall of algae with molecular weight of average 20KDa weighing 5–20% by dry weight. The source of fucoidan is from brown algae and brown seaweed such as undaria pinnatifida, cladosiphon okamuranus, focus vesiculosis and ascophyllum nodosum. Fucoidan consists of α 1-3 linked sulphated L fucose with repeating sequence of alternating α 1-3 and α 1-4 glycosidic bonds.

19.2.2.1 Structure

Fucoidan derived from marine echinoderms has anticoagulant and antithrombotic activities, acts as a ligand for either L or P selections like heparin or heparin sulphate and is active on cell growth, migration and adhesion (Fig. 19.9).

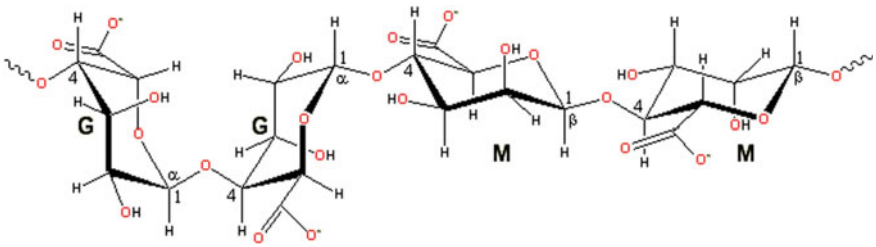

Fig. 19.9 Polymeric and isomeric structure of fucoidan

Fucoidan from seaweeds bind and modulate activity of proangiogenic growth factors such as fibroblast growth factor. In the presence of growth factor fucoidan enhances the nature of endothelial cells, decreases plasminogen activator inhibitor-1 release and regulates cells surface.

19.2.2.2 Extraction Process

The extraction process involves three stages (Fig. 19.10).

19.2.2.3 Isolation (Alternate Process)

An alternate extraction and purification process for fucoidan is shown in Fig. 19.11.

Factors affecting the properties of fucoidan include:

1. Reaction time, purity of chemicals, ambient conditions;
2. Rheological features and viscosity are dependent on inherent factors of algae and seaweeds; size and species; and
3. Bioactivity is related to molecular size, type of sugar, sulphation degree and molecular geometry.

19.2.2.4 Biopharmaceutical Applications

Application of fucoidan in biopharmaceutical industry include anticoagulant, antiviral, and immunologic activities.

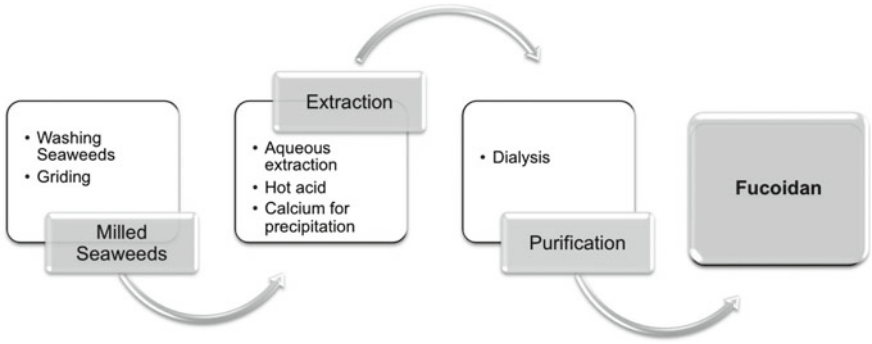

Fig. 19.10 Fucoidan extraction and purification process

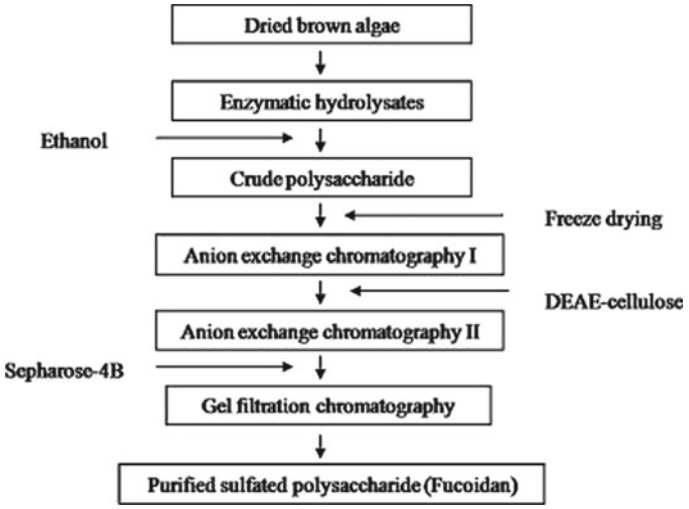

Fig. 19.11 Alternate extraction and purification process for fucoidan

Anticoagulant Activity

The anticoagulant and antithrombotic activities of sulfated fucans. The algal and invertebrate sulfated fucans have potent anticoagulant activity, mediated by anti-thrombin and/or heparin cofactor II. This aspect was clarified as studies were extended to invertebrate polysaccharides. These definitively established that regular, linear sulfated α-L-fucans and sulfated α-L-galactans express anticoagulant activity, which is not simply a function of charge density, but depends critically on the pattern of sulfation and monosaccharide composition [28]. The anticoagulant activity of sulfated polysaccharides is achieved mainly through potentiation of plasma cofactors, which are the natural inhibitors of coagulation proteases. The conformational activation of anti-thrombin and the consequent formation of a covalent complex with thrombin appear to be less important for the anticoagulant activity of sulfated galactan than for heparin. Their results demonstrate that the paradigm of heparin-anti-thrombin interaction cannot be extended to other sulfated polysaccharides. Each type of polysaccharide may form a particular complex with the plasma inhibitor and the target protease [29].

Antiviral Activity

Fucoidans demonstrated their antiviral activities by mainly blocking the interaction of viruses to the cells so as to inhibit viral-induced syncytium formation [30]. Isolated fucoidans from several species such as: *Adenocytis utriclaris*, *Undaria pinnatifida (Mekabu)*, *Stoechospermum marginatum*, *Undaria pinnatifida*, and *Cystoseira indica*

[29], exhibited potential antiviral effects againstHSV-1 and HSV-2 deprived of cytotoxicity for Vero cell cultures. Elizondo-Gonzalez and colleagues [31] reported that the isolated fucoidan from *Cladosiphon okamuranus* showed potent antiviral activity against Newcastle disease virus in the Vero cell line at the initial stages of infection. The viral-induced-syncytial formation declined by exposure of fucoidan prior to cleavage of the fusion protein, which led to attachment of fucoidan to the protein. Consequently, fucoidan exhibited a better antiviral potency than ribavirin. Moreover, fucoidan can effectively augment immune system health by activating immunoreactions of the cellular and humoral types and by increasing macrophage phagocytosis [32]. In conclusion, fucoidan directly affects the secretion of extracellular matrix proteins, influences the proliferation of cells, and can activate apoptosis [33].

Antioxidant Activity

A number of studies has revealed that fucoidan presents significant antioxidant activity in experiments in vitro. It is an excellent natural antioxidant and has great potential for preventing free radical-mediated diseases [34]. Fucoidan from *L. japonica* can prevent the increase of lipid peroxide (LPO) in serum, liver and spleen of diabetic mice obviously. However, no inhibition effect was found on both spontaneous lipid peroxidation of homogenates and that induced by Cys/$FeSO_4$ in vitro [35]. Antioxidant activity relates to the molecular weight and sulfate content of fucoidan. Fucoidan fractions from *L. japonica* had excellent scavenging capacities on superoxide radical and hypochlorous acid, except the highly sulfated fraction L-B. In LDL oxidation system, low molecular weight fractions L-A and L-B exhibited great inhibitory effects on LDL oxidation induced by Cu^{2+}, however F-A and F-B had little inhibitory effects in this system due to their large molecular weights [36]. Both molecular mass and sulfate content of fucoidan played very important roles in the effects on the azo radicals 2-2′-Azobis(2-amidinopropane) dihydrochloride (AAPH) induced LDL oxidation [37]. The correlation between the sulfate content and scavenging superoxide radical ability was positive, the ratio of sulfate content/fucose was an effective indicator to antioxidant activity of the samples [38].

19.2.2.5 Biomedical Applications

Tissue Engineering

Fucoidan in combination with polymers such as chitosan, alginate or polycaprolactone processed into hydrogels, scaffolds, films and nanofibers which supports the cell system. Fucoidan blended with polyesters, PCL following melt plotted process provides appropriate pore structure for bone regeneration.

Once fucoidan is combined with heparin binding growth factors FGF-1 and FGF-2, low molecular weight chitosan enhances the activity of growth factors, helps matrix formation of fibrous collagen, assists fibroblast proliferation, stimulates angiogene-

sis, influences osteoconductive properties like ALP activity, collagen type 1 expression and mineral deposition thereby inducing bone cell proliferation.

Furthermore, the osteogenic differentiation of fucoidan enhances expression of osteogenesis specific marker genes (ALP, osteopontin, type 1 collagen, RUNX-2 osteocalcin).

Fucoidan Composites for Bone Tissue Engineering

Cell proliferation and alkaline phosphatase (ALP) activity is improved in biocomposites composed of fucoidan with PLGA (polylactic glucolic acid) and PCL (polycaprolactone). Fucoidan is a sulfated polysaccharide that contains L-fucose and sulfate. It is commonly found in marine brown seaweeds. Fucoidan can increase the level of ALP, type-1 collagen expression, osteocalcin and BMP-2 and even helps in mineral deposition associated with mineralization [39]. In addition, fucoidan treatment enhanced the expression of ALP, type-1 collagen, Runt-related transcription factor 2 (Runx-2), osteopontin and osteocalcin in human adipose-derived stem cells. It also promoted osteogenic differentiation in human amniotic fluid stem cells, which suggested that it is a potential candidate for bone tissue regeneration [40]. Composite containing polycaprolactone-fucoidan showed excellent cellular proliferation and mineralization. Around 30% enhanced mineral deposition was observed in fucoidan containing composite. This is mainly because of the presence of fucoidan in the composite scaffold. Moreover, Chi-fucoidan composite film showed significant wound dressing ability in vitro and in vivo [41].

Lee et al. [42] have developed electrospun polycaprolactum (PCL) composite with fucoidan (1–10% wt.) scaffold. It has various beneficial biological functions, including anticoagulant, antiviral, and immunomodulatory activity. The resultant electrospun composites exhibited improved tensile modulus and strength for limited weight fractions (<10 wt%) of fucoidan when compared with the pure PCL fibre mats.

Drug Delivery Applications

Fucoidan along with chitosan form a micro complex and serves as a carrier for release of fibroblast growth factor (FGF). Fucoidan induces vascularisation and tissue formation. Nanoparticles act as nanocarriers and help in the sustained release of drug for tissue engineering purposes. The structural and anionic characteristics of fucoidan are similar to those of heparin. Heparin stimulates production of hepatocyte growth factor (HGF), which has key roles in tissue regeneration [42]. Fucoidan and fucoidan-derived oligosaccharides have similar ability to stimulate production of HGF as heparin and heparin-derived oligosaccharides. This induction of HGF by heparin or fucoidan and their oligosaccharide derivates occurs primarily at the level of translation, probably via the same mechanism. Fucoidan may thus be useful

to protect tissues and organs from various injuries and diseases, via mechanisms involving HGF [43].

In recent years, therapeutic angiogenesis has been proposed in the treatment of chronic ischemia. In animal studies, it was shown that basic fibroblast growth factor (FGF-2), which is mitogenic for vascular endothelial cells, fibroblasts, and smooth muscle cells, can induce angiogenesis in vivo [44]. High-molecular-weight (HMW) fucoidans are known to bind with growth factors such as FGFs and protect them from proteolysis. A fraction of low-molecular-weight (LMW) fucoidan (7 ± 2 kDa) was obtained by radical depolymerization of HMW extracts from brown seaweed and was devoid of any direct anti-thrombin effect. LMW fucoidan can also promote therapeutic revascularization, potentiate FGF-2 activity, mobilize stromal-derived factor (SDF)-1, and facilitate angiogenesis in a rat model of critical hindlimb ischemia. This natural compound could be of interest as an alternative for conventional treatment in critical ischemia [45, 46].

Would Healing

Fucoidan has potential for use in wound and burn treatment; it has heparin-like activity triggers transforming growth factor (TGF-β1) while providing fibroblast migration and activation in damaged tissue. Second-degree dermal burns are one of the most common pathologic conditions. These kinds of burns involve the loss of deep dermis; healing starts at damaged dermal areas, beginning with significant scar formation (epidermal thickening) and functional losses and generally show aesthetic deterioration and pigmentation changes by the end of treatment. In the study of fucospheres and chitosan microspheres, it is shown that, due to free amine groups in the structure of chitosan, the composite possess bioadhesive properties. With the increase of fucoidan, bioadhesion of the microparticles also increased. The reason for this increase was that negatively charged sulfate groups in the structure of fucoidan had an ionic interaction with the proteins in the wound area. The treatment effectiveness of fucosphere formulation was investigated on superficial burns. It was found that at the end of 14 and 21 days, the fucosphere treated group had the highest contraction area and thus the highest healing level and that treatment effectiveness of fucospheres was higher than those of chitosan microparticles and fucoidan solution. However, in another studies, it was indicated that the treatment effectiveness of combined fucoidan-chitosan formulations (microsphere, hydrogel and film) was higher in comparison to formulations containing chitosan alone [47].

19.2.2.6 Advantages and Limitations

The advantages of fucoidan include:

1. Efficient and resilient class of biosorbents;
2. Selective metal binding; and

3. Stimulates expression of osteoblastic markers differentiation such as alkaline phosphatase activity, collagen type I expression and mineral deposition, also cell proliferation.

However, the disadvantages of using fucoidan include:

1. Fabrication of scaffolds is difficult due to its solubility in water; and
2. Limited information on fucoidan-based system used in bone tissue engineering scaffold and wound healing.

19.2.3 Laminarin

Laminarin is a storage polysaccharide representing 35% algal by dry weight. It is a straight chain polysaccharide comprised of 25–50 glucose units linked by β 1,3 glycosidic bonds and β 1,6 glycosidic bonds in few places, the ratio being 3:1. The source of laminarin is from Laminaria Saccharina and ascophyllum, undaria and focus species. The content differs seasonally; it neither forms gel or viscous solution.

Laminarin has application in medical and pharmaceutical industries. It has anti-tumour and anticoagulant activity and is used as dusting powder [48].

19.2.4 Carrageenan

19.2.4.1 Introduction

Carrageenans are sulphated polysaccharides from red algae that occurs as a matrix material in several species of red seaweeds Rhodophyceae. The most abundant source is from chondrus crispus, gigartina, eucheuma cottonii and spinosum. Carrageenans represents 60–80% of algae along with proteins, floridean starch and various other smaller compounds. Carrageenan consists of alternating three-linked β-D-galactopyranose and four-linked α-galactopyranose.

Carrageenans are categorized according to the number of sulphate groups per disaccharide unit into three groups: kappa (κ), iota (ι), and lambda (λ) bearing 1, 2 and 3 sulphate units respectively. All types are water soluble at low temperatures. Kappa carrageenan is a good gelling agent, while lambda carrageenan is non-gelling and is a good thickener. Lambda carrageenan contains D-galactose and its mono and bisulphate esters. A decrease in the sulphate content will result in harder the gels formation. This gelling property attributes to broad field of application.

19.2.4.2 Structure and Isolation

The basic structure of carrageenan is disrupted by a more or less ordered distribution of sulphate hemi ester groups. Carrageenan can also contain some methoxy and pyruvate groups (Fig. 19.12).

Carrageenans are distinguished from agars in that the B units in carrageenan are in the D form whereas they are in the L form in agars. Gelling in carrageenan is the result of helix formation and this can only occur in repeat structures where the B residue is in a 1-C-4 conformation. Lambda carrageenan has both its sugar residues in a 4-C-1 conformation and does not form gel. All the gelling types of carrageenan which include 'κ' and 'ι' all contain a 3,6 an hydro bridge on the B unit which forces the sugar to flip from a 4-C-1 conformation to a 1-C-4 conformation and can then form cross-link networks and gels (Fig. 19.13).

19.2.4.3 Applications

Carrageenans have been widely researched for drug delivery, tissue engineering and wound healing [49].

Tissue Engineering

This polysaccharide is mainly used as combination with various materials in tissue engineering applications:

1. Carrageenan combined with graphene composite stimulates hydroxyapatite nucleation and increases adhesion, morphology and proliferation of cells;
2. Carrageenan along with chitosan is used for bone tissue regeneration;
3. Carrageenans with growth factors helps in regeneration and enzyme immobilization;
4. Carrageenan based hydrogel encapsulates cell proliferation and increases chondrogenic differentiation of stem cells [50].

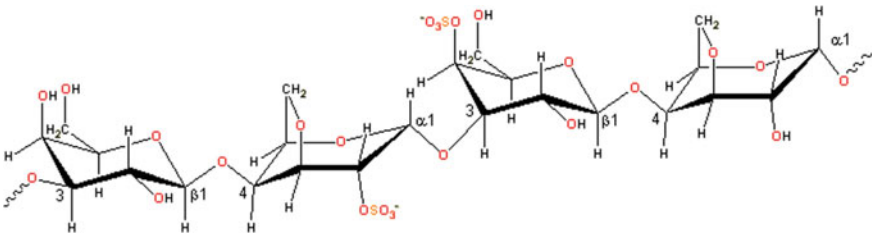

Fig. 19.12 3-D structure of Carrangeenan

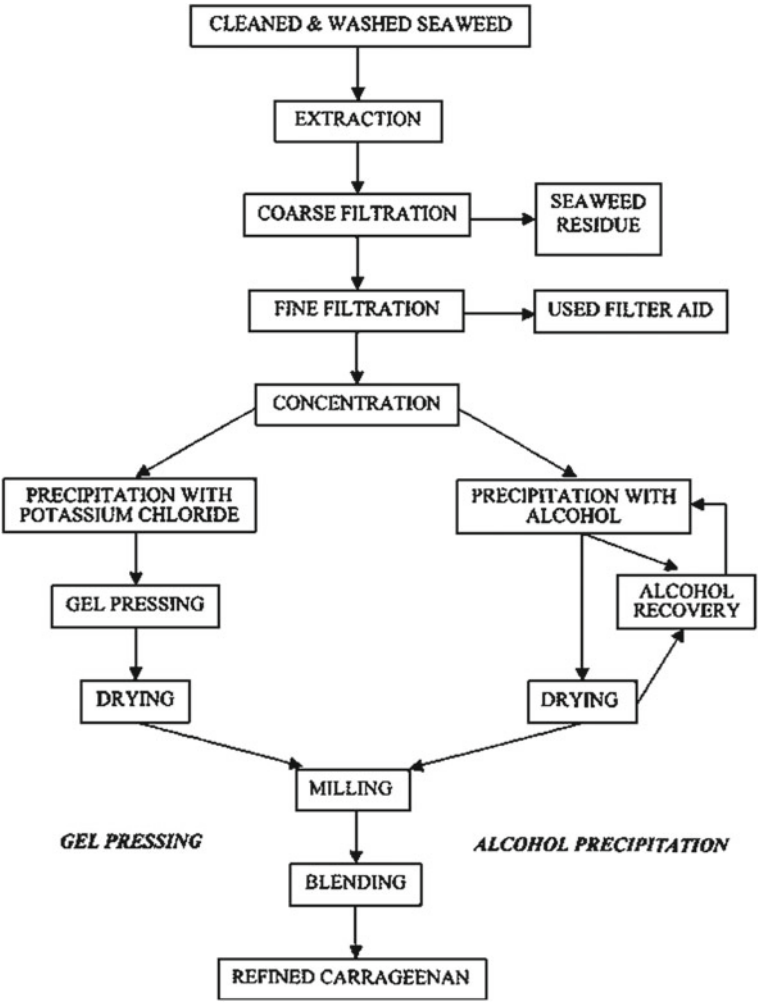

Fig. 19.13 Isolation of Carrageenan

Drug Delivery

1. Carrageenans are available in different shapes and sizes in tablets, as stabilizing, gelling agent and beads. They deliver sustained release of the drug;
2. Carrageenan with chitosan also have sustained release in treating hypercholesterolemia. They show low toxicity on cells;
3. K carrageenan targets NSAIDs to treat the infection and inflammation. These encapsulate cell and growth factors [51].

Wound Healing

1. 'K' carrageenan hydrogels are known to yield good mechanical properties, biocompatibility and prevention of infection which are prerequisites for wound healing [52].

Other Applications

Carrageenan is also used as a biosensor. They are also used in treating respiratory illness, common cold to influenza virus, antibacterial activity [53]:

1. Used as thickening agent, emulsifier, gelling and stabilizing agents;
2. In water treatment to remove heavy metals and ions; and
3. Antioxidant activity against fungal and bacterial infections.

19.2.4.4 Advantages and Limitations

The advantages of using carrageenans include their antiviral activity and their ability to bind to metals. Their gelation properties are also advantageous.

However, the drawback of carrageenan is their questionable biocompatibility. Carrageenan is mainly employed as food additive and pharmaceutical excipient, but they have some limitations. Toxicological properties of Carrageenan are as follows: LD50 (rat, oral) > 5 g/kg; LD50 (rabbit, skin) > 2 g/kg; 4 h LC50 (rat, inhalation) > 0.93 mg/L [52]. Even though Carrageenan is non-toxic, due to the presence of the sulfate group, carrageenan exhibits adverse effects towards blood coagulation and immune system. The presence of sulfate groups on G-6 causes the strongest cytotoxicity. Additional care is required when formulating Carrageenans into blood contact biomaterials, for example, tissue recovery scaffolds or parental drug delivery vehicles. Hence, it is necessary to perform more epidemiological and vital studies to assess the safety of Carrageenans [54].

19.2.5 Ulvans

19.2.5.1 Introduction

Ulvans are extracted from the cell wall of green seaweeds, they account for 8–29% of algal dry weight. Ulvans are derived from Ulva luctuca which is a water-soluble polysaccharide. Ulvans include glucose, rhamnose, xylose,glucoronic acid, iduronic acid and sulphates. Basically, ulvans are composed of L-rhamnose and sulphates linked to D-glucoronic acid.

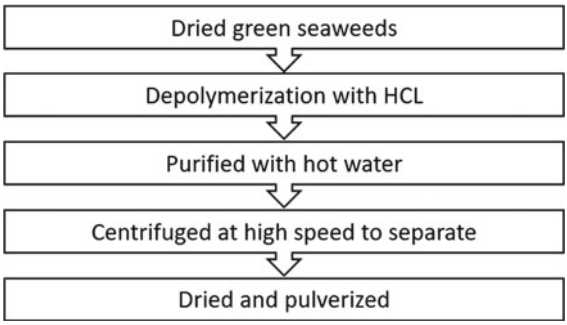

Fig. 19.14 Ulvans extraction process flow diagram

19.2.5.2 Extraction Process

Ulvans are extracted from cell walls of green seaweeds and depolymerised using HCl at 100 °C for 45 min which releases mainly monosaccharide a disaccharide unit. The suspension is then centrifuged at 10 °C for 20 min at 10,000 rpm to remove solid particles to obtain ulvans, which are finally dried and pulverised (Fig. 19.14).

19.2.5.3 Applications

Unlike other marine polysaccharides, ulvans also have scope in various domains, and they are being tested for application in tissue engineering and drug delivery and have shown promising potential in biomedical field.

Tissue Engineering

Once ulvan hydrogels/scaffold are incorporated with enzymes/growth factors, these biocomposites will assist in the achievement of osteogenic differentiation thereby increasing mineralization potential [55]. Furthermore, chemically altered ulvan with chitosan mimics the extra cellular matrix and is known to enhance cellular functions and finally induce regeneration. Enzymatically altered ulvans induce ALPase activity and increase mineralization potential.

Drug Delivery

Ulvans have been used as a novel drug delivery mode in wound healing and in treating hyperlipidemia. In addition, ulvans exhibit antioxidant, anti-tumour, immuno mediator, anti-inflammatory and anticoagulant activities. Ulvans have great potential in other fields like food, agriculture, pharmaceutical and industrial applications [56].

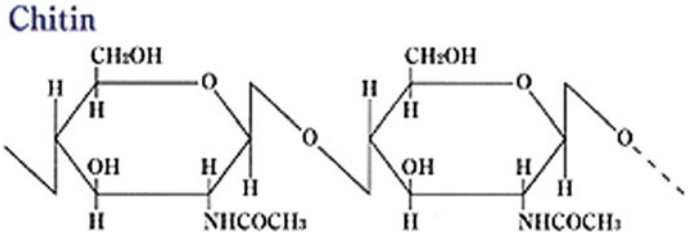

Fig. 19.15 Structure of chitin

19.2.5.4 Advantages and Limitations

As a result of its chemical structure similar to that of natural glycosaminoglycans such as chondroitin sulphate and hyaluronic acid, this makes ulvan an attractive candidate for their substitution or use in related applications.

However, they are limited to certain applications as a result of their weak mechanical properties and their uncontrollable dissolution in presence of physiological fluids.

19.3 Marine Crustaceans: Shells from Shrimps, Crabs or Squilla Fish

19.3.1 Introduction to Chitin and Chitosan

Chitin is a linear polysaccharide comprised of units of 2-acetamide-2-deoxy-β-d-glucopyranose linked through β-(1,4) bonds (Fig. 19.15). This polysaccharide has a structure similar to cellulose but instead of having a hydroxyl group at carbon number 2, chitin has the N-acetyl group. Chitin has average molecular weight (MW) of between 1.03×10^6 and 2.5×10^6 Da and is a polysaccharide which is insoluble in water as well as in most organic solvents. Chitin can be dissolved in solutions with high ionic strength such as hexafluoroacetone or dichloroethane with mineral acids and 5% lithium chloride in dimethylacetamide.

Chitosan is a deacetylated product of chitin composed of 70–90% D-glucosamine and 10–30% N acetyl glucoamine linked by glycosidic bonds. Chitosan has higher degree ofdeacetylation units which makes it soluble in acetic acid but insoluble in water. In an effort to enhance its solubility in water, it is treated or altered by deacetylation, sulphation and carboxymethylation. Deacetylation is important and is vital to physicochemical and biological properties of chitosan. Chitosan with varying degree of polymerization has various chemical and biological properties that make it versatile for several biomedical applications. The chitosan easily forms a crosslink to be used as scaffold material and binds with human tissue growth (e.g. Glutaraldehyde, hyluronic acid, heparin, gelatine, aloe vera etc.) [57].

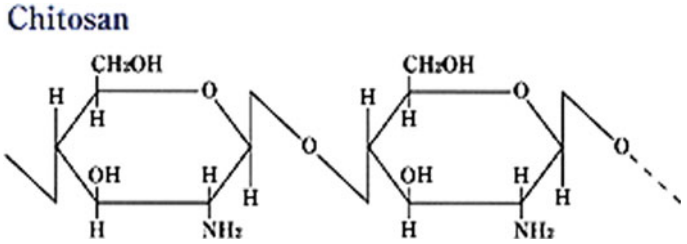

Fig. 19.16 Structure of chitosan

As a primary marine polysaccharide, chitosan has evolved to become a potential source of effective tissue engineering material over the years. Due to the presence of reactive groups, it has special properties such as bioactivity, antimicrobial activity, biocompatibility and compatibility to blend with other materials [57].

Chitosan is obtained via partial or total chitin deacetylation and can be classified as a copolymer of 2-amino-2-deoxy-β-d-glucopyranose (glucosamine) and 2-acetamide-2deoxy-β-d-glucopyranose (N-acetylglucosamine) (Fig. 19.16). In general, if the content of N-acetyl groups is greater than 50%, it is considered chitin; and if it is lower than 50%, then it is considered as chitosan. It has a nitrogen content of 6.80 to 7.4% and is characterized by molecular weights between 1×10^5 and 5×10^5 Da [57] (Fig. 19.17).

19.3.2 Isolation and Production

Chitosan is isolated from the cell wall of prawn, crab, shrimp or Squilla. Isolation is possible by different techniques like chemical, enzymatic and Fermentation process [57, 58]. Production of chitosan can be carried out via chemical means (Fig. 19.18). The basic steps involve:

1. Decalcification—by HCl solution;
2. De-proteination—by NaOH solution;
3. Discolouration—0.5% potassium permanganate or oxalic acid; and
4. Deacetylation—by 50% NaOH

The advantages of chemical production are:

1. Short processing time; and
2. Quality material for wide variety applications, for example water treatment, agriculture, paper, pharmaceutical, dental and medical fields.

On the other hand, there are limitations, such as:

1. The chemical method is not environmentally friendly, due to the large amount of alkaline waste and organic material generated during the process;

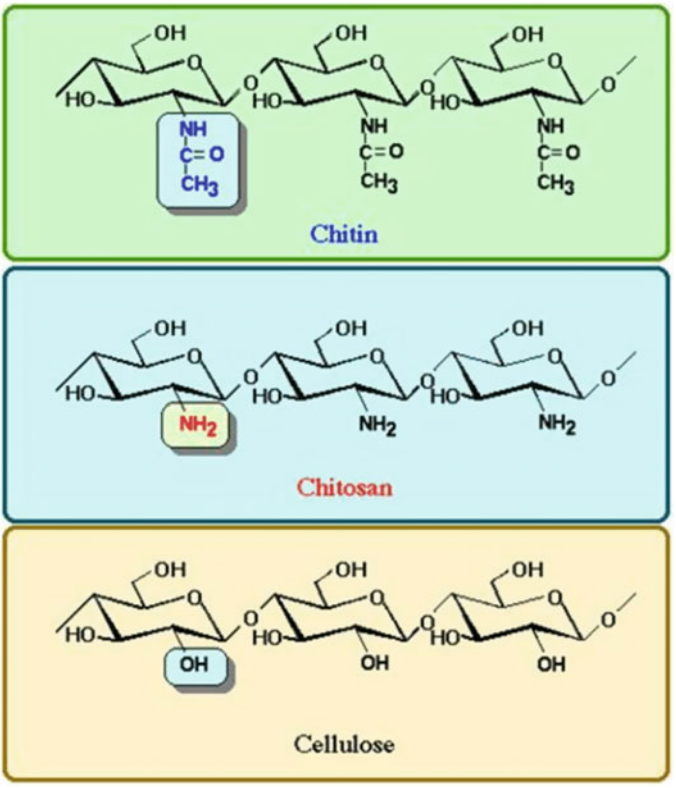

Fig. 19.17 Structural comparison between chitin, chitosan and cellulose

2. Expensive due to of the effluent generated costs for its treatment and disposal, acidic and alkaline effluents, unless the unit has upward integration to produce chitosan derivatives; and
3. The continuous hydrolysis of the polymer during the alkaline treatment causes a decrease in the molecular weight and therefore its mechanical properties.

19.3.2.1 Chemical Modifications

Chitosan derivatives have desirable properties like water solubility, degradation, compatibility and its efficacy for wide range of applications. Furthermore, chitosan possesses the following advantages:

1. It can be doped with synthetic polymers;
2. The properties of chitosan can be engineered simply by changing its size and shape—micro and nano size particles increase the properties for diffusion and provide interaction at desired site;

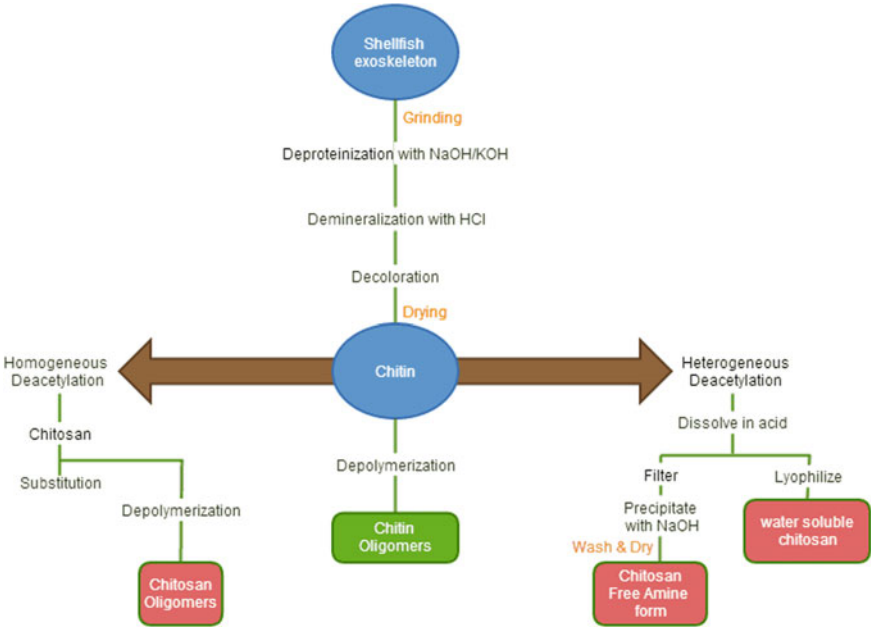

Fig. 19.18 The production of chitosan and its derivatives using the chemical method

3. Cross linking with various chemical agents increases its biocompatibility; and
4. High degree of deacetylation also increases its biological and biomedical properties.

Chitosan can be modified chemically using the following methods:

- **Carboxyalkylation**: This results in the formation of water-soluble chitosan which exhibits polyelectrolyte nature suitable for biomedical applications. Carboxyl and amino chitosan exhibit biophysical properties suitable for drug delivery (antibiotic drug release) [59].
- **Sulphonation**: Chitosan on sulphonation exhibits solubility, mostly disrupts crystallinity and the resultant micelles aid in drug delivery [59].
- **Acetylation**: Chitosan when acetylated with acids and esters exhibits antifungal properties, increases solubility, protein absorption and drug delivery [59].
- **Sugar modified chitosan**: sugar modified derivative acts as a matrix for cell attachment, increases cellular functions and regenerative potential [60].
- **Graft copolymerized chitosan**: Chitosan when combined with composites or ceramics is known to improve complex formation, retention and absorption and improves the biological functions making the medium a suitable yield for biomedical application [60].

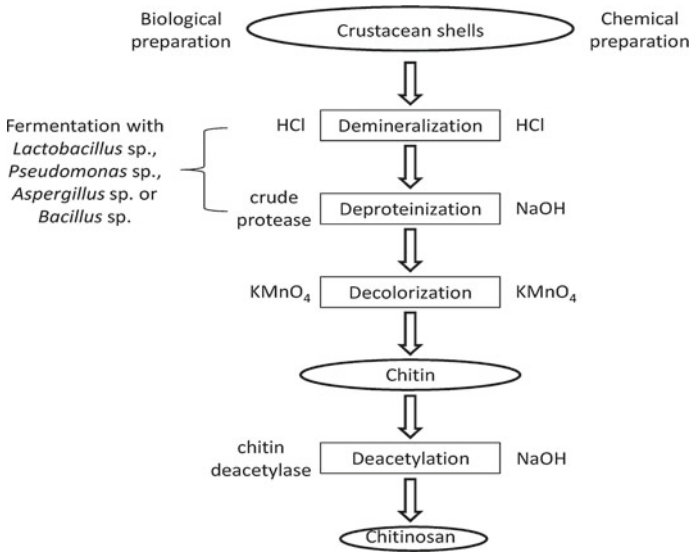

Fig. 19.19 Chitosan production by enzymatic alteration

19.3.2.2 Enzymatic Production

This method is an alternative to chemical production method as discussed above. Chitosan may be produced by enzymatic alteration method with enzymes like alkalise, chymotrypsin and papain. However, industrial scale production is not feasible due to long process time [61]. In this method, bacteria producing proteolytic and chitinolytic enzymes have been used and tested with optimal fermentation temperature of 30 °C and supplemented with 10% glucose.

One key advantage of producing chitosan using this approach in comparison to the chemical method, is the process is very eco-friendly. Unfortunately, this method is generally not the preferred method as the cost associated with the production of chitosan is extremely high (Fig. 19.19).

19.3.3 Properties and Characteristics

Chitosan is a natural, cationic biopolymer and it has been used in personal care products for skin lotions and shampoos. Chitosan as a dietary supplement for weight reduction is known for many years. Due to its improved anti-microbial properties the chitosan composition may be used for wound care including providing healing promotion, microbial contamination prevention, blood gelation and scarring prevention. The compositions can be especially useful for the treatment of burns due to the anti-microbial and film forming properties. Chemical modification of chitosan has

Table 19.1 Properties of chitosan

Properties	Values
Appearance	Off-white—flaks/powder
Degree of deacetylation	>85%
Moisture	6–8%
Heavy metals	<10 ppm
Proteins	<0.2%
Ash	<0.2

Table 19.2 Characteristics of chitosan and their methods of determination

Physicochemical characteristics	Method of determination
Molecular weight	Viscometry; gel permeation chromatography; light scattering; high performance liquid chromatography; matrix-assisted laser desorption/ionization-mass spectrometer
Degree of deacetylation	Infrared spectroscopy; ultra violet spectrophotometry; nuclear magnetic resonance spectroscopy (1H-NMR and 13C-NMR); conductometric titration; potentiometric tiltration; differential scanning calorimetry
Crystallinity	X ray diffraction

Table 19.3 Difference between chitin, chitosan and chito-oligo saccharides

	Molecular weight	Solubility	Absorption properties
Chitin	MW more than 1000 kDa	Insoluble in water, acid, alkali, solvents	Poor absorption
Chitosan	MW > 100 kDa	Insoluble in water but readily soluble in acids such as acetic acid, HCl, lactic acid	Absorbability about 1–3%
Chito-oligo saccharides	MW < 2 kDa	Completely dissolves in water	Absorbablity almost 100%

two main aims: (a) to improve the metal adsorption properties, and (b) to change the solubility properties of chitosan in water or acidic medium. The substitution chemical reactions involve the NH_2 group in the C_2 position or the OH groups in the C_3 and C_6 positions of acetylated and deacetylated units.

There are a number of factors which can have an influence on the stability of chitosan, for example the purity, molecular weight, moisture content and the degrees of acetylation and polymerisation. The pattern related to the degree of acetylation will also influence the stability of chitosan. In addition, external factors such as the processing method, sterilization, and environmental issues such as humidity and temperature will also affect the stability (Tables 19.1, 19.2 and 19.3).

19.3.4 Applications of Chitosan

According to a study by Vunain et al. [62], chitosan can be used for the following applications:

- Pharmaceutics [63]:
  1. Gels and hydrogels (for controlled sustained drug release);
  2. Films and membranes (for controlled drug release);
  3. Emulsions (microspheres and microcapsules for sustained drug release, increased bioavailability, and mucoadhesion);
  4. Targeted cancer therapy (retention and accumulation of drug in tumour); and
  5. Systems for controlled delivery/release of peptide drugs, vaccines, genes.
- Medicine and biomedicine [64]:
  1. Wound dressings, wound treatment, bandages;
  2. Sutures and surgical implants;
  3. Heamodialysis membranes and biomedical devices coatings; and
  4. Heamostatics.
- Tissue engineering [65]:
  1. Scaffolds, hydrogels, artificial skin grafts
- Other non-medical applications [66]:
  1. Agriculture, food industry, textile industry, waste water treatment, cosmetics.

19.3.4.1 Processing Chitosan for Tissue Engineering Applications

Being flexible, chitosan can be moulded into different shape, forms and size. Depending on their intended use in the biomedical arena, chitosan has been developed and processed into various forms such as membranes, scaffolds, sponges, hydrogels, beads, films, microgels, nanoparticles and nanofibers for tissue engineering, drug delivery and biosensors (Fig. 19.20) [67].

19.3.4.2 Biomedical Applications of Chitosan and Chito Oligosaccharides

The versatility and favourable biological properties of chitosan polymer have made them ideal for use in a wide number of applications. Chitosan has been used for many biological and biomedical applications and hence it has remained as a key interest in research, development and commercialization. Individually, chitosan lacks bioactivity and in order to improve its bioactivity, it has been used to produce with bio-composites/cross-linked to enhance this property [64].

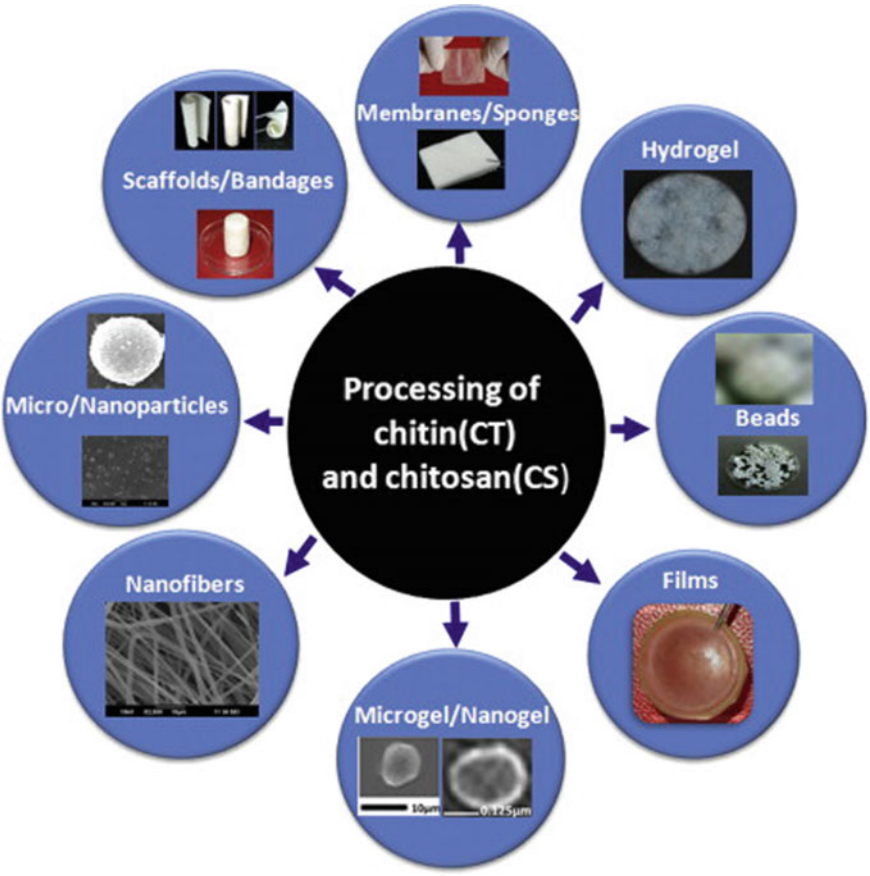

Fig. 19.20 Chitosan products for biomedical and tissue engineering applications

Antimicrobial, Anti-inflammatory, and Anticoagulant Activities

Chitosan is a positively charged molecule and the target of its antimicrobial action is the negatively charged cell wall of bacteria, where it binds and disrupts the normal functions of the membrane, e.g., by promoting the leakage of intracellular components and also by inhibiting the transport of nutrients into the cell [68]. The antimicrobial activity of chitosan is well known against a variety of bacteria and fungi coming from its polycationic nature.

Anti-bacterial activity also increases with increasing molecular weight of chitosan, though too high a molecular weight or concentration is counterproductive.

The antimicrobial activity of chitosan was observed against a wide variety of microorganisms including fungi, algae, and some bacteria. However, the antimicrobial action is influenced by intrinsic factors such as the type of chitosan, the degree

of chitosan polymerization, the host, the natural nutrient constituency, the chemical or nutrient composition of the substrates or both, and the environmental conditions.

The chitosan antimicrobial activity is more immediate on fungi and algae than on bacteria. Chitosan has been shown to be fungicidal against several fungi. The minimum inhibitory concentrations (MICs) reported for specific target organisms range from 0.0018 to 1.0% and are influenced by a multitude of factors such as the pH of the growth medium, the degree of polymerization of chitosan, and the presence or absence of interfering substances such as lipids and proteins.

Bactericidal efficacy of chitosan is achieved due to microbial antimicrobial factors, intrinsic factors like density, molecular weight, concentration, chelating potential, physical state and environmental factors. The amino groups in chitosan interacts with on the cell wall and disrupts the transport of materials across the cell wall leading to bactericidal act [69].

Photo cross-linked electrospun mats containing quaternary chitosan (QCS) were efficient in inhibiting growth of Gram-positive bacteria and Gram-negative bacteria [69]. These results suggested that the cross-linked QCS/PVP electrospun mats are promising materials for wound-dressing applications. A polyelectrolyte complex (PEC), which consists of chitosan as a cationic and γ-poly (glutamic acid) (γ-PGA) as an anionic polyelectrolyte, was developed as a wound dressing material [70]. Chitosan/alginate composite membrane incorporated with ciprofloxacin HCl had the potential for wound dressing application [71]. A biocompatible carboxyethyl chitosan/poly(vinyl alcohol) (CECS/PVA) nano-fibers were prepared by electrospinning of aqueous CECS/PVA solution as wound dressing material. Medical gear for doctors and para-medical staff who work very close to patients can wear this antimicrobial chitosan coated fabric clothing while on duty or at their workplace. This can be a preventive measure for bio safety application for field staff on their jobs during epidemic outbreaks [72].

In terms of anti-inflammatory activity, chitosan circulating through blood increases cytokines and mediates immune system, inhibits PGE2 and alters inflammatory response [73].

With respect to its anticoagulant activity, chitosan that has been chemically treated with sulphates increases the clotting factor levels and shows anticoagulant activity. Sulphated chitosan prolongs thrombin and clotting time [74].

Drug Delivery

Attempts were made by researchers to develop a safe delivery vehicle based on chitosan. The targeted drug delivery system is comprised of three components: a therapeutic agent, a targeting moiety, and a carrier system. The drug can be either incorporated by passive absorption or chemical conjugation into the carrier system. The choice of the carrier molecule is of high importance because it significantly affects the pharmacokinetics and pharmacodynamics of the drugs. Currently chitosan, cationic polysaccharide, is drawing an increasing amount of attention within the pharmaceutical and biomedical arenas, owing to its abundant availability, unique mucoadhesiv-

ity, inherent pharmacological properties, and other beneficial biological properties such as biocompatibility, biodegradability, non-toxicity and low-immunogenicity. The chemical modification of chitosan imparts amphiphilicity, which is an important characteristic for the formation of self-assembled nanoparticles, potentially suited for drug delivery applications. The hydrophobic cores of the nanoparticles could act as reservoirs or micro-containers for various bioactive substances. Because of their small size, nanoparticles can be administrated via the intravenous injection for targeted drug delivery. Conjugation of the targeting moieties to the surface of drug-loaded nanoparticles may improve therapeutic efficiency of the drug. Chitosan has been widely utilized as drug delivery systems for low molecular weight drugs, peptides and genes. Chitosan is administered orally and parenterally to tackle gram positive and gram-negative bacteria, virus and fungi. Chitosan entraps the molecules through crosslinking and controls drug release. Higher the molecular weight of chitosan imparts there is delayed/sustained drug delivery to target site and which improves the therapeutic efficacy. Drug delivery is attained in the form of tablets, beads, hydrogels, scaffolds, vaccines, microspheres [72].

Anticancer Drug and Antioxidant Effects

Chitosan has been displayed to show anticancer activity by stimulating macrophage and suppressing the tumour growth. Chitosan removes hydroxyl, alkyl, DPPH radicals at free NH2 site and exhibits stability. Methylated chitosan has higher mechanical properties and can be framed into desirable shapes and size for effective antioxidant activity [73].

19.3.4.3 Tissue Engineering Applications of Chitosan

Chitosan is being explored in regenerative medicine to provide bio-functions to repair damaged tissues like bone, cartilage, skin and muscles. Tissue engineering provides mechanical support and compatibility in tissues through matrix, scaffold and cells (Fig. 19.21). These channels act as an artificial extracellular matrix reabsorbed by the body until new tissue is formed [73].

Wound Healing

Wound healing bandages have been developed and commercialized during last 10 years or so. It is mainly using chitosan with medium to low molecular weight compounds. Films which are formed using this molecule have shown good oxygen permeability, moisture retention and wound healing properties. These films are made in the form of bandage to form cuts/injuries. Company in the USA, Tricol Biomedical (as discussed in Sect. 19.4.1) produces these wound healing bandages commercially [74].

Chitosan has been found to have an accelerator effect on wound healing/wound dressing process. Chitosan may be used to inhibit fibroplasia in wound healing and to promote tissue growth and differentiation in culture [75]. Biological features of chitosan yield intense activities as described in Fig. 19.22.

During the degradation of chitosan, fibroblasts are proliferated. The degradation also promotes collagen deposition and hyaluronic acid stimulation at wound site which increases healing without scar formation [75].

Chitosan in the form of nanoparticles increases the physical, mechanical and biological properties and helps in faster healing as the adherence to cells is increased. Chitosan-based dressing interacts with blood cells, the positively charged chitosan binds with negatively charged red blood cells (RBCs) and form clot over wound and induces heamostasis. Commercially, many chitosan-based dressing products are available like chigel, chitopad, chitoflex, chitogauge (as discussed in Sect. 19.4.1).

A study by Burkatovskaya et al. [74] investigated the effect of using HemCon® bandage in wound healing in mice. This bandage is an engineered chitosan acetate

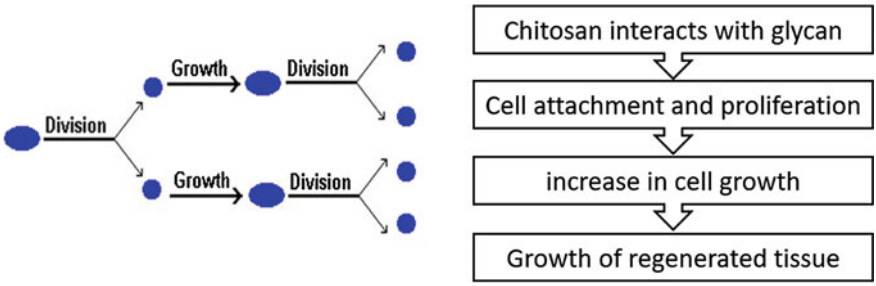

Fig. 19.21 Interaction of chitosan during tissue growth

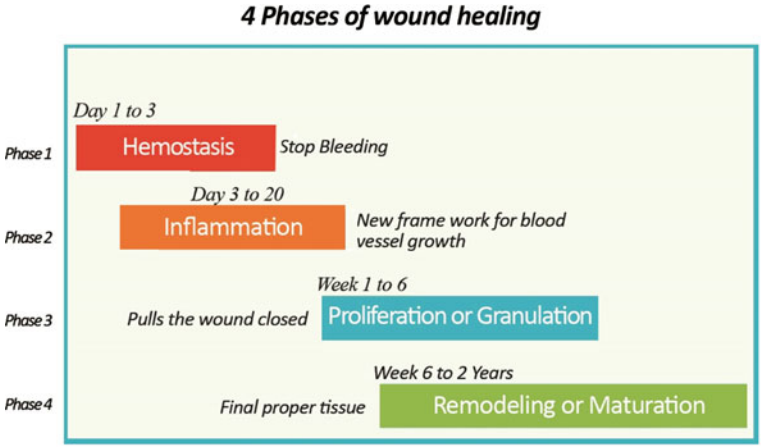

Fig. 19.22 The phases associated with wound healing

preparation designed as a haemostatic dressing and is under investigation as a topical antimicrobial dressing. The effect of the bandage application on excision wounds that were or were not infected with *Staphylococcus aureus*, in normal mice or mice previously pre-treated with cyclophosphamide (CY). Chitosan acetate bandage reduced the number of inflammatory cells in the wound at days 2 and 4 and had an overall beneficial effect on wound healing especially during the early period where its antimicrobial effect is most important. Chitosan film was used as a wound dressing in male Wister rats [76]. The results suggested that chitosan film treatment might have beneficial influence on the various phases of wound healing such as fibroplasias', collagen synthesis and contraction resulting in faster healing. It was possible that the enhanced healing of wounds in rats by chitosan film was a result of its stimulating activity and/or its capacity to stimulate fibroblast proliferation resulting in the progression of wound healing. It was also suggested that chitosan might induce fibroblasts to release interleukin-8, which is involved in migration and proliferation of fibroblasts and vascular endothelial cells [77–79].

Obesity Treatment

Chitosan is soluble in the acidic conditions of the stomach and forms a gel when the molecular weight is high. When fat and chitosan in the diets are eaten together, the viscous chitosan entraps the fat droplet in the stomach [77].

Chitosan has been shown to decrease serum cholesterol in animal and human studies. When animals were fed for 20 weeks on a diet containing 5% chitosan or on a control diet, the blood cholesterol levels were significantly lower in the chitosan fed animals throughout the study, and at 20 weeks were 64% of control levels. This study was the first to show a direct correlation between lowering of serum cholesterol with chitosan and inhibition of atherogenesis and suggests that the agent could be used to inhibit the development of atherosclerosis in individuals with hypercholesterolaemia [78].

19.3.4.4 Chitosan in Bone Tissue Engineering

Table 19.4 summarizes the outcomes of a number of studies carried out over the past decade or so on the use of chitosan for bone tissue engineering applications. Various studies have been conducted to assess the efficacy of chitosan when used in biocomposites. These studies have tested the type of chitosan and its effect on mechanical and biological properties. All the studies have proved that chitosan has enhances cell attachment, increased ALP activity thereby increasing the mineralization resulting in bone tissue regeneration In addition to bone tissue engineering, investigations were also carried out on the feasibility of utilizing chitosan for wide range of dental applications such as oral surgery (Table 19.5), and other branches of dentistry (Table 19.6).

Table 19.4 Recent studies on chitosan-based material for bone tissue engineering applications

Scaffold content/Pore size	Chitosan type	Method of preparation	Mechanical properties	Cell culture studies/Type of stem cells used	References
PLGA/nHA, CS/pDNA nanoparticles (encoding BMP-2) Particle size: 100 nm	Medium MW CS (DD: 75–85%)	Electrospinning Membrane (Microfiber)	Increased tensile strength	High cell attachment and high cell viability, increased DNA release when CS nanoparticles before electrospinning; Cytotoxic and high transfection efficiency when CS nanoparticles added after electrospinning Human marrow stem cells	[80]
nHA/CS Pore size: 45–125 μm	Medium MW CS (250 kDa, DD: 75%; High MW CS 400 kDa, DD: 83%)	Lyophilization Sponge	Induced compression modulus	Increased cell attachment, 1.5 times greater cell proliferation	[81]
nHA, CS/gelatin Particle size: 17–25 nm	CS (MW 2.0×10^2 kDa, DD: 85%)	Biomineralization Film	Not studied	Good biocompatibility, increased cell attachment and proliferation, allowed osteogenic differentiation Mesenchymal stem cells	[82]
CS/poly(butylene succinate), CS nanofibers Particle size: 500 μm	Medium MW CS (DD: 85%)	Electrospinning Membrane (Microfiber)	Increased tensile strength, no significant difference in tensile stress	Not studied	[83]

(continued)

Table 19.4 (continued)

Scaffold content/Pore size	Chitosan type	Method of preparation	Mechanical properties	Cell culture studies/Type of stem cells used	References
PLA, CS microspheres, BMP2 derived synthetic peptide Pore size: 100–300 μm	CS (MW: 2.0×10^2 kDa, DD: 90%)	Lyophilization Sponge	Significantly increased compressive strength and modulus	Not studied	[84]
CS, biphasic calcium phosphate Pore size: 100 μm	CS (800 kDa, DD:>85%)	Lyophilization Sponge	Porosity: 80%	Cell attachment and spreading, more prominent actin cytoskeletons, significantly higher ALP activity and osteocalcin production. Mouse mesenchymal stem cells	[85]
CS (drug loaded)	Medium MW CS (DD: 85%; Source: Sigma Aldrich)	Lyophilization Sponge (loaded with supercritical fluid technology)	Porosity: 87%	Not studied	[86]
CS/PLGA microspheres Particle size: 500–710 μm	CS (DD: 83.3%; Source: Vanson HaloSources, Inc., USA)	Sintering by heating	Significantly increased compressive modulus and compressive strength; decreased porosity with increasing sintering temperature. Porosity: 28–37%	Significantly increased ALP activity, no significant difference in cell proliferation, significantly higher osteopontin and bone sialoprotein. Mesenchymal stem cells	[87]

Table 19.5 Chitosan applications in dentistry

Dental specialities	Applications	Types of Research	References
Preventive dentistry	(a) Oral hygiene supplements (Mouth wash, dentrifice, hypersensitivity); and (b) Mucoadhesive cariostatic substance delivery systems	In vivo and in vitro	[88–91]
Conservative dentistry	(a) Direct pulp capping (b) Indirect pulp capping (c) Antibacterial against *s mutans*; and (d) Gingival retraction cord coated with chitosan	In vivo and in vitro	[92–96]
Endodontics	(a) Root canal irrigant (b) Removal of smear layer; and (c) Antibacterial	In vitro	[97–103]
Oral surgery	(a) Guided bone regeneration; (b) Wound healing; and (c) Haemostasis	In vivo	[104–108]
Periodontology	(a) Guided periodontal tissue engineering (b) Antioxidant delivery system (c) Antibacterial (d) Scaffold in periodontal tissue engineering	In vitro In vivo In vivo In vivo and in vitro	[109–114]

19.3.4.5 Other Biomedical Applications

In addition to the applications discussed above, chitosan is also used in the treatment of medical problems such as:

1. Treatment of cardiovascular diseases.
2. Shown to lower triglyceride levels.
3. Assist in maintaining degenerative diseases.
4. Auto immune diseases as it increases the immunity.
5. Dry mouth syndrome.

Table 19.6 Summary of studies carried out on chitosan-based materials for periodontal tissue engineering

Scaffold content/Pore size	Chitosan type	Method of preparation	In vitro mechanical properties examination	In vitro cell culture studies/Types of cells used	In vivo cell culture studies	References
CS/collagen, CS/pDNA nanoparticles (encoding PDGF) Particle size: 30–40 nm; Pore size: 200–300 μm	CS (DD:>5%; Source: Sigma Aldrich)	Lyophilization Sponge with nanoparticles	High degree of interconnectivity; Porosity:>90%	Improved growth rate and proliferation, formation of periodontal tissue-like structure; PDL cells	Not Studied	[115]
CS/coral (calcium carbonate), pDNA (encoding PDGFB) Pore size: 200–300 μm	CS (DD:>85%)	Lyophilization Sponge	Not studied	Significant cell proliferation, no cytotoxicity, significantly increased mRNA expression levels of PDGFB HPLCs	Athymic mice Sampling: Day 2, Week 4 (Applied with HPLC cells) No inflammation, increased expression of PDGFB, cell proliferation, new vascular tissue growth	[116]
HA beads (bFGF)CS Particle size: 40 μm Pore size: 20–100 μm	CS (DD:>5%; Source: Sigma Aldrich)	Lyophilization Sponge	Interconnected structure	Significant increase in the cementoblast and PDL cell counts, well organized F-actin meshwork, higher mineralization, significantly higher alkaline phosphatase activity PDL cells, cementoblasts	Not Studied	[117, 118]

(continued)

Table 19.6 (continued)

Scaffold content/Pore size	Chitosan type	Method of preparation	In vitro mechanical properties examination	In vitro cell culture studies/Types of cells used	In vivo cell culture studies	References
CS/taurine	Chitosan-H (DD: 80%, MW: 1400 kDa)	Solvent casting Film	Not Studied	Not Studied	Beagle dogs Cellular activity observed both in the mitochondria of fibroblasts and macrophages	[119]
CS/collagen/demineralized bone matrix	(MW: 252.000 Da; DD: 84%)	Gelation Gel Membrane	Not Studied	Not Studied	Human Sampling: 0,3, 6 months Statistically significant bone fills compared to Day 0	[120]
CS/bovine type I collagen/Plasmid and virus encoding TGF-ß1 gene Pore size: 200 μm	Chitosan (DD: 85%; Source: Sigma Aldrich)	Lyophilization Sponge	Interconnected structure Porosity: 80%	Better proliferation HPLCs	Athymic mice Sampling: week 2 higher transfer efficiency	[121]

19.3.5 Advantages, Problems, and Limitations Currently Associated with the Use of Chitosan and Chitin

Chitosan possesses a number of advantages, these include:

1. Good biological properties;
2. Chitosan is flexible can be moulded into any shape, form and size; and
3. Wound healing, antioxidant, antimicrobial, anti-tumour, cytotoxicity.

However, their uses may be limited due to the facts that they are:

1. Not soluble in organic solvents;
2. Insoluble in water; and
3. Stability is questionable.

Chitin is a typical naturally derived bio-polymer, and the large-scale production of chitin is limited due to the seasonal availability of crabs and shrimps. These are either sweet water cultivated or directly obtained from open sea fishing activity. The natural material must be processed in a short time frame. There is also a variation in the quality of chitin produced based on different geographical location across the world. Value added produced derived from chitin can vary from batch to batch. It has been discussed in international forums and standardization is being carried out to maintain a standard of quality. There are other issues related to environmental pollution due to usage of high alkali levels and its disposal, unless it is recycled however cost of neutralization is high. There are also soluble calcium salts unutilized or discarded during the processing. This water-soluble calcium has high market value, however very few processing units carry out the separation. Similarly, high HCl concentration can be utilised for production of ZnCl2, by reaction with metallic zinc powder. This material is used for primary cell production instead of just neutralizing acid.

19.4 Future Prospects in Marine Polysaccharides

1. Development of appropriate matrix with acceptable physical and biological properties is a challenge for success of tissue engineering.
2. Despite the fact that numerous studies have been carried out in the past using both animal and human models to examine the feasibility of utilizing chitosan as a bone filler/regenerative material, the non-uniform study design and material selection have not set the standards in adopting an evidence-based practice to prove the efficacy. Lack of accuracy/consistency in clinical evaluation has hampered their success rate.
3. Ethical issues for carrying out histomorphological and radiological analysis poses a challenge, which questions the efficacy of the biomaterial.
4. The combination of both natural or crosslinked biopolymers with synthetic polymer are ideal matrix materials as they mimic the bone structure. The unique and

superior properties of chitosan have made it the material of choice for tissue engineering, along with the fact that chitosan is highly flexible and can be moulded in any form and economical when compared to other grafts. Hence, they are considered as a material of choice for bone substitute.
5. Chitosan possesses osteogenic potential when used with mesenchymal stem cells and displays properties very similar to bone, hence this material needs to be investigated with appropriate design criteria based on these evidences.

19.4.1 Commercially Available Products

Tissue engineering is a novel field in biomedical science that intends to fully initiate the original processes to fix damaged tissues and cells. Haemorrhage is the most common cause of death for severely injured patients when prompt action was not taken within a critical time period. Haemorrhage control and subsequent treatment is important for survival the patient. A number of products have been commercialized in the past 15–20 years.

Four European Directives and an ISO specification regulate the requirements for marketing and putting into service medical devices:

1. Active Implantable Medical Devices (AIMDD) Directive 90/385/EEC—OJ L189/20.7.90;
2. Medical Devices Directive (MDD) Directive 93/42/EEC—OJ 169/12.7.93;
3. In Vitro Diagnostic Directive (IVDD) Directive 98/79/EC—OJ331/7.12.98;
4. Directive 2007/47/EC; and
5. ISO specification 10993 Part 1 to 5—Biological evaluation of Medical Devices: Evaluation and Testing published in 1995 provides guidelines for various test methods and evaluation criterion for new products introduction in the market.

Some of the companies offering commercial products are listed below:

- http://www.tshs.eu/en.html
- https://www.medline.com/
- https://www.sammedical.com
- www.umtmedical.com/
- https://www.tricolbiomedical.com/
- www.anscare.benqmaterials.com
- www.axiobio.com
- www.everestbio.com/applcations/dental.

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Part IV
Marine Sources for Soft Tissue Engineering and Other Biomedical Applications

Chapter 20
Bioactivity of Red Sea Algae for Industrial Application and Biomedical Engineering

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Abstract The Red Sea is largely undiscovered, strange wellspring of bioactive materials that its waters have not received sufficient and broad inspection till date. It expands for approximately 2000 kms and its semi segregation jointly with a rising saltiness at high water temperatures have offered new research trends for biological communities and developmental adjustments. Just a couple of marine green growths have been accounted for from the Red Sea up until now (27 passages in Algae Base rather than 512 for the Caribbean and 307 for the Arabian Gulf) and researches exploring the bioactivity of Red Sea algae are not many. Marine living beings have ended up being a rich wellspring of unprecedented and hopeful bioactive atoms for an extensive variety of uses, including novel therapeutics, cosmetics, and biotechnological applications. Marine algae, beside whichever benthic organisms, are predominately influenced by marine biofouling (epibiosis). It was confirmed that the aforementioned creatures yield secondary metabolites with antialgal, antibacterial, antifungal, anti-macrofouling and antiprotozoal characteristics in order to retain their surfaces without epibionts. Accordingly, natural yields from marine algae prove to be a favourable alternate source of unprecedented ecologically friendly compounds. Interestingly, exposing algae to light and high oxygen concentrations will stimulate the formation of inflammatory mediators like ROS and NOS. Consequently, algae are capable to produce the substantial compounds in order to protect themselves from external factors like UV radiation, stress and pollution. Generally, natural products are the main origin of compounds utilized in cancer therapy with over 75% of antineoplastic drugs in clinical research trials being either acquired or at least created by nature. Hence, marine algae possess a particular function since they are to an increasing extent significant dietary constituent in considerable portions of the world and are discussed as prospective, pharmaceutical foods in cancer management. In addition to

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the anti-cancer activity, marine algae display a multitude of anti-viral activities with an essential number of investigations concentrating on the human immunodeficiency virus type 1 (HIV-1), thereby exploring the significance of the aforementioned viral pathogen. HIV-1 keeps being a considerable human being health concern since there are more than 35.3 million people infected globally and 2.3 million new infections annually. Algal compounds were found to attack different steps of HIV-1 replication, covering viral entry and the main viral enzymes such as Reverse Transcriptase (RT), Integrase, and Protease. Furthermore, epidemiological discoveries indicate a consumption connection of marine algae with a low prevalence of HIV/AIDS in several Eastern Asia locations.

Keywords Macro/micro algae · Seaweed · Alginate · Polysaccharide · Bioactive · Vitamins · Regenerative medicine · Pollution

20.1 Introduction

Algae are global; they are not only abundant in oceans and seas, but they also grow in fresh water streams like rivers lakes or ponds. Moreover, they are very capable of growing on other moist surfaces such as rocks, soil, snow, ice and even plants and animals. Algae photosynthesis consists about 40% of the whole ubiquitous process. Algae are considered as microscopic plants which are extremely diverse; their size ranging from 1/1000 to 2 mm and they are split into four groups: (i) brown algae, (ii) green algae, (iii) red algae, and (iv) diatom [1]. They exist as macroalgae, microalgae and cyanobacteria. The classification is illustrated in Table 20.1.

Microalgae are peculiar. They are photosynthetic organisms that can be prokaryotic or eukaryotic. They are capable of growing in a broad domain of ecological conditions [1].

In contrast, macroalgae (seaweed) are multicellular photoautotrophic organisms and are classified into three main categories; Chlorophyta (green algae), Rhodophyta (red algae) and Ochrophyta-Phaeophyceae (brown algae). In addition to the role of these organisms as primary producers, they serve significantly in marine ecosystem structuring and preservation.

20.2 The Red Sea Algae

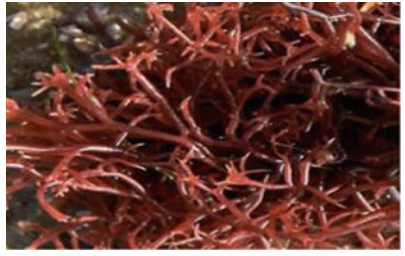
So far, Red Sea algae characteristics and bioactivities are less investigated and just few information regarding their particular activities were communicated. In the coral reefs of Red Sea, marine algae are exposed to many defiance including space competition high salinity, high water temperatures in addition to intensive sun radiation. Such drastic circumstances have forced these organisms to evolve various adapta-

Table 20.1 Seaweed and cyanobacteria taxonomy and applications [2]

Family name	Classification	Application	Image
Cyanobacteria	Known as: blue-green algae	Generation of renewable energy by converting sunlight into electricity	
Phaeophyceae	Known as: brown algae between 1,500 and 2,000 species are known worldwide	All brown algae contain alginic acid (alginate) in their cell walls, which is extracted commercially and used as an industrial thickening agent in food and for other uses	

(continued)

Table 20.1 (continued)

Family name	Classification	Application	Image
Rhodophyta	Known as: red algae, one of the largest phyla of algae, containing over 7,000 currently recognized species	Several species are important food crops, in particular members of the genus *Porphyra*, variously known as nori (Japan), gim (Korea), or laver (Britain). Dulse (*Palmaria palmata*) is another important British species. These rhodophyte foods are high in vitamins and protein and are easily grown; for example, nori cultivation in Japan goes back more than three centuries	
Chlorophyta	Known as: green algae, informally called chlorophytes. They include about 7,000 species	Green algae are commercially important as feed and as a source of many industrial and pharmaceutical chemicals	

tional possibilities in order to be able to survive; this adaptability gave rise to the development of wealthy algal bioactive compounds [3].

Red Sea was first explored in the 18th century when the 1st algal record was published by Strand [4]. Then, the first considerable algal gathering was made by Forsskal in 1775 during his journey to Arabia and Egypt where new specimens were described and investigated by botanists then revised by Borgesen in 1932. Consequently, more than 500 benthic algal taxa were discovered from the Red Sea. The northern and central areas of the Red Sea are characterized by seasonally abundance of small brown, filamentous green, and tuft-forming (calcareous-coralline) red algae in the shallow coral reefs. While the southern areas of Red Sea are characterized by the abundance of perennial brown algae, like *Hormophysa, Cystoseria* and *Sargassum* that present over shallow hard substrates. Usually, the hard substrates are mainly covered by macroalgae where these areas appear very turbid due to coral growth. Unfortunately, there is no detailed information or clear background about the conditions and situations of the Red Sea algal communities, but it is known that algal reefs in Farasan Islands might be built under low energy environment by calcareous algae on rugged deposits at depth between 2 and 4 m [5].

20.3 Chemical Composition

Owing to the significantly high nutritional value, seaweeds are conventionally utilized worldwide particularly in Asian countries as a food source [6, 7]. Food and Agriculture Organization of the United Nations (FAO) statistics assures that global seaweed production to be as much as 15.1 million tonnes with a value of 7.2 billion US dollars in 2006 [8]. At the present time, the seaweed is exploited as nutrition in particular, since spices and delicacies are outspreading in western countries as a consequence of the alteration of the way of living and dietary habits. The purpose of scientific attention toward marine algae is not only due to the nutritive value as appropriate food industries constituent-like brown seaweed *Undaria pinnatifida* [9], but also due to its high content of bioactive compounds that have been widely used in the preparation of both medicinal and pharmaceutical products [10].

However, economic importance of seaweed is due to their exploitation as raw material in the manufacturing of hydrocolloids—agar, carrageenan from red seaweed and alginates from brown seaweed. They are widely implemented in cosmetics, food as well as in pharmaceutical and medical industries. Genera of agar-consisting of red seaweed *Gelidium sp*. and *Gracilaria*; genera of carrageenan-consisting of red seaweed Chondrus crispus, Gigartina, Iridaea, Kappaphycus alvarezii and *Euchema denticulatum* are utilized extensively [6]. Excluding from the best-known species, that are aforementioned, lesser-recognized species are in the scientific objective of hydrocolloids production like green algae *Cladophora* spp. [11].

Previously, seaweed employment as animal feed was localized as well in coastal European states particularly, Norway, Ireland, Iceland, Spain, Portugal, and France [6, 12]. Furthermore, seaweed extracts found their way as plant growth supplements.

This is attributed to their cytokinin content [13]. Cytokinin is known as a substantial factor of the plant growth and evolution. Furthermore, it enhances cell division and motivates shoot proliferation [14]. *Ascophyllum nodosum* which is, *Laminaria* spp. and the robust alga named as *Fucus serratus* are utilized as organic and mainstream fertilizers in Europe [6, 15] for several diversities of crops. This is attributed to its incorporation of both micronutrients (Ca, Mg, S, Mn, Zn, Fe, etc.) and macronutrients (K, P, and N). The chemical composition of marine algae satisfies their high nutritional value participating to human being nutrients—like proteins with all substantial amino acids, vitamins, minerals, organic acids, polysaccharides, etc.

Moreover, they contain bioactive secondary metabolites beside several compounds with health benefits [16]. Seaweeds contains slight quantities of lipids. The later are predominately polyunsaturated ω-3 and ω-6 fatty acids. Seaweed has a high dietary fibre that does not belong to the nutritional factors. However, it has protective impact on human health. On the other hand, certain species of seaweed can contain native toxic compounds that may limit the application of that seaweed as food or animal feed, for instance, *Caulerpa taxifolia* contains sesquiterpene caulerpyne that blocks the mitotic cycle of sea urchin embryos and also inhibits stimulation of mitogen-activated protein kinase. Moreover, caulerpyne displays growth-inhibitory impacts in certain human cancerous cells. Furthermore, its effect on the nerve cells activity has also been reported [17].

The chemical composition of seaweed differs, based on the species sorts, collection time, geographic habitat, and on several external situations like nutrient concentration in water, light intensity and water temperature [18]. A number of studies were conducted to examine the variable composition of seaweed. They present considerable nutritional factors variations. The aforementioned variations are namely, proteins, minerals, lipids, or dietary fibre. They are specified within various genera of seaweed [18–20]. It has been widely accepted that in the same genus of seaweed there are enormous differences in their constituents [21, 22].

20.3.1 Proteins and Amino Acids

Proteins are factors of immense and significant importance setting up the nutritional value of food. Their biological values are established on the sufficient quantities of substantial amino acids. Seaweeds own considerable quantities of the aforementioned nitrogenous compounds. Moreover, they contain little quantity of non-protein nitrogen, which is the source for several compounds like nucleic acids, nitrate and nitrite nitrogen, free amino acids, chlorophyll, and ammonium ions [23]. The potential to use seaweed as a source of proteins has continued for many decennium. This is attributed to protein deficiency, particularly in third world states. For now, seaweeds are turning into inexpensive alternate origin of proteins. Protein concentration in seaweeds alters in accordance with the factors like different species, environmental conditions. Moreover, it is dependent on the implemented approach of protein determination [23–25]. A seasonal impact on the nitrogen content in red seaweed

Palmaria palmata from the French Atlantic coast was first published by Fleurence [25], followed by Galland-Irmouli [26]. The largest nitrogen content was recorded in winter and early spring seasons from February to May, as well as in November. On the other hand, the lowest amount was observed during summer and autumn from July to October. Comparable findings of changeable nitrogen content of different seaweed species have also been examined and reported [25, 27].

The protein amount also differs with respect to the amount of the nitrogen-to-protein conversion factors used. Generally, protein content in seaweed is calculated by multiplying seaweed nitrogen value, (it is defined by the Kjeldahl method which is also called Kieldahl digestion), by a nitrogen-to-protein conversion factor. Nevertheless, protein nitrogen and non-protein nitrogen are determined by Kjeldahl digestion. The classical value of the conversion factor is 6.25 for animals (likewise for several plant proteins). It is determined on the hypothesis that protein contains 16% of nitrogen. Nonetheless, this demand is not complete in several seaweed. This is due to the existence of various non-protein nitrogen content. Thus, protein content results might be overvalue employing the factor of 6.25 for seaweed containing larger quantities of other nitrogen compounds like phospholipids, free amino acids, non-protein amino acids, amines, amides, nitrites, and vitamins. Otherwise, protein content results can be underrated in seaweed that contains lesser quantities of non-protein nitrogen compounds.

20.3.2 Minerals

Minerals are substantial fractions of human diet. This is attributed to the deficiency of the human body to produce them. More than 95% of mineral taken into the body is created from food. Therefore, the mineral level in human beings depends on mineral concentricity in vegetable or meat used as raw ingredients for food products. Minerals have significant functions in the human body since they are structural materials for building tissues. Moreover, they are regarded as considerable factors in vital reactions, as well as being cofactors of several metalloenzymes [28–32].

Seaweeds are famous for being a significant origin of minerals. This is attributed to their absorption capability of the inorganic substances from the environment. This capability is due to the existence of polysaccharides in seaweed cell walls. Furthermore, it is capable to predetermine a place of mineral storage in several parts of seaweed tissue [33]. A number of parameters could affect the storage and distribution of minerals in seaweed. The aforementioned parameters are namely based on various environmental conditions such as wave exposure, seasonal effects, and geographic locations. Further parameters are conditions of seaweed such as age [19, 34]. Moreover, the chemical forms of minerals and their background levels in seawater may influence the absorption of minerals by seaweed [35]. Therefore, the accumulation of certain minerals by seaweed can be greater than the concentration of the same elements in the surroundings by many orders of magnitude [36]. It

was observed that *Laminariales* have a significantly high capability of accumulating iodine by more than 30,000 times the iodine concentration in the seawater [37].

The existence of various polysaccharides in seaweed cell walls explains why different groups of seaweed differ in minerals uptake capability. The higher metal sorption capacity of brown as compared to red and green seaweed possibly attributed to the high presence of alginic acid, alginate, and salt of alginic acids. These polysaccharides, that are included in seaweed cell walls predominately like sodium, calcium, potassium and magnesium salts, have strong ion exchange characteristics [38, 39]. Some minerals accumulation mechanism in various species of seaweed has been documented. Nevertheless, adsorption investigations were carried out largely in the connection with recognition of toxic metal ions uptake mechanism by various species of seaweed [21, 38, 40]. Additionally, brown seaweeds cell walls that are generated by cellulose and its carboxyl groups may contribute to the accumulation of metals [38].

20.3.3 Vitamins

Vitamins are organic macromolecules that significantly influence several metabolic activities in humans. Indeed, autotrophic organisms are capable of synthesizing vitamins, while heterotrophic organisms including humans can synthesize vitamins in a very limited extent. Thus, vitamins are essentially obtained from dietary sources including seaweed that provide an important source of several lipid-soluble and water-soluble vitamins.

20.3.3.1 Water-Soluble Vitamins

Water-soluble vitamins in seaweed principally include vitamins of the B group, particularly B1, B2 and B12 and vitamin C. Due to the high abundance of L-ascorbic, a biologically active form of vitamin C, seaweeds have been considered and involved in scurvy prevention for an extended period of time [41].

20.3.3.2 *Fat-Soluble Vitamins*

Seaweed provides a major resource of lipid-soluble vitamin E and carotenoids (as provitamins of vitamin A). Vitamin E is one of the ultimate significance lipid-soluble vitamins with robust antioxidant properties. Its particular role is to protect the lipid membrane from peroxidation. It has eight different forms: α, β, γ, δ-tocopherols and α, β, γ, δ-tocotrienols. The α-forms demonstrate the most vigorous antioxidant activity [42]. Furthermore, there is a correlation between vitamin E and hypertension reduction [43]. Brown seaweed contains higher amounts of vitamin E than red and green seaweed.

Carotenoids are also very potent antioxidants. Both red seaweed and green algae have the same carotenoids composition, which includes α- and β-carotene and their derivates like zeaxanthin and lutein. While the principle brown seaweed carotenoids include β-carotene, lutein, violaxanthin, anteraxanthin, zeaxanthin and neoxanthin [44], fucoxanthin is the major carotenoid of brown seaweed, which demonstrates several beneficial health effects. Its chemopreventive and antitumor activity in human leukemia cells was proven through the induction of apoptosis [45]. Furthermore, fucoxanthin was prescribed as an anti-obesity and anti-diabetic agent [46, 47].

20.3.4 Lipids

Seaweed contains very limited amounts of lipids ranging from 1 to 5% of dry seaweed weight. Nevertheless, the lipids majority are polyunsaturated ω-3 and ω-6 fatty acids that efficiently minimize the danger of osteoporosis, diabetes and cardiovascular diseases [46]. Eicosapentaenoic and docosahexaenoic (DHA) polyunsaturated ω-3 fatty acids have shown their efficiency in cardiovascular protection, through reducing triacylglycerol and cholesterol levels in addition to their anti-inflammatory and anticancer effects [46]. Generally, the lipid level and composition of seaweed fatty acids is affected by various environmental circumstances like varying light intensities and seawater salinity.

20.4 Marine Algae as a Source of Industrial Exploitable Compounds

Marine algae significantly contribute to vast array of daily life applications; not only as a primary food source for different ecosystems on our planet but they also provide marine-derived bioproducts which are therapeutically effective due to their pharmacologically active content [48, 49] representing multiple cytotoxic, antifungal, antiviral as well as anti-inflammatory and antioxidant capacities [50–52]. Thus, vigorous investigative researches have been carried out to obtain bioactive compounds from marine origins as natural sources of drugs [53].

Marine algae produce a vast diversity of distinguishable natural components known as secondary metabolites that don't affect the basic machinery of life [54]. However, they provide a primary role for the organism integrity and survival such as growth and reproduction in spite of the fact that these molecules predominantly contribute to extremely tiny portion of the organism total biomass [55]. The noticeable halogenation of numerous secondary metabolites reflects the abundance of chloride and bromide ions in seawater. These two halogens are the mostly utilized ones to enhance the bioactive properties of secondary metabolites whilst fluoride and iodine appear to be less commonly included within these chemical compounds [56] although

chlorine concentrations are higher than those of bromine, marine algae usually use bromine more frequently to produce organ halogen. Thus, all marine halogenated compounds such as phenols, indoles, peptides, terpenes, polyketides acetones as well as volatile halogenated hydrocarbons [57] have their own bioactive properties that render the compound interestingly applicable in different industrial fields.

20.4.1 Pharmaceutical Industries

Alginic acid (alginate) is a polysaccharide that exists in the algal cell wall and abundantly present in brown algae cell wall. This component has been widely introduced together with its salts into the pharmacological products. Alginates are especially important during the production of concentrated emulsions, gels and suspensions due to its binding capacity as a stabilizer and thickening agent. Thus, it has been used as a basic component in creams and ointments [58]. In conjunction with other drugs, sodium alginate can be applied on septic wounds as the polysaccharide base enhances healing and prepare wound for scarring [59]; for example, "Algipore" which is a lyophilized gel consisting of sodium alginate, calcium alginate and *Furacillin* has antimicrobial and wound reparative effects. It has been hypothesized that calcium alginate releases calcium ions that promote fibroblast proliferation and keratinocytes motility without affecting the microvascular endothelial cells and thereby promote wound healing [60]. Moreover, high-molecular sodium alginate-based solutions are usually applied in the form of lotions or wet dressings as a protective coating on injured skins or mucous membranes to prevent further irritation from the surrounding environment. Interestingly, these solutions are also applicable to conjunctiva serving as artificial tears. Other applications include its role as anti-irritative beverage for the gastrointestinal tract as well as its applications in enemas [60].

20.4.2 Dentistry

Marine algae compounds provide many beneficial contributions in stomatology. Furans, which consist of a variety of polysaccharides and sulfated galactans, show their efficacy in minimizing the opportunity for dental caries incidence in rats by preventing oral bacterial adherence and colonization [61]. Calcium alginate is another essential component representing hemostatic characteristics by which alginate fibers are capable of imbibition and turning gelatinous as soon as they are applied to the tooth surface. This gelatinous nature acts as a coagulant matrix which renders alginate applicable as a dressing material in packing the tooth cavity as well as fistulas and sinuses [62]. Moreover, an alginate-based drug "polaprezinc-sodium alginate suspension" is produced as specific treatment for severe gingivostomatitis associated with hemorrhagic erosions and ulcers. This drug acts via two principle mechanisms: first, sodium alginate serves as a hemostatic agent and secondly, polaprezinc in addi-

tion to its ability of binding the free radicals, it stimulates healing and reparation of the mucous membrane [63].

In prosthetic dentistry, alginates are essentially used as impression materials to produce the negative impressions of intraoral structures for the purpose of diagnosis as well as construction of restorative appliances such as fillings, crowns, dentures and orthodontic apparatus [64].

20.4.3 Surgery

Alginates have been introduced into the surgical field principally as hemostatic agents. Specific surgical materials such as cotton, gauze and swabs are impregnated with sodium alginate solution and used for both external hemostasis as a packing material for burns, wounds or nasal bleeding [65] and for internal hemostasis such as in the case of parenchymatous organs operations [66]. For instance, "Alto", which is a drug based on sodium alginate and they are utilized for uterocervical hemorrhagic conditions due to its adhesive and hemostatic efficacy [67]. Calcium alginate swabs also demonstrated a profound hemostatic effect when applied intraoperatively during adenoidectomy and during internal fixation of femoral fractures [68].

20.4.4 Anticancer Activities

Multiple marine algal derived bioactive metabolites exhibited excellent anticancer activities. Some algae are considered as the chief resource of such bioactive compounds as they are consumed as the main dietary object in some regions. Micro algae (marine blue-green algae or cyanobacteria) are considered as one of the richest sources of bioactive substances which exhibit very potent antineoplastic efficacy by either enhancing cancerous cells apoptosis or by activating some members of protein kinase-c family enzymes which influence the cell signaling [69]. The anticancerous roles of microalgal compounds are summarized in Table 20.2.

Macro algae (seaweed) are also considered as an important source of various nutritive components including vitamins, proteins, iodine and minerals in addition to its wealthy content of polyphenols like gallic acid, epigallocatechin, epicatechin and catechin. Hence, their metabolites have demonstrated very promising antioxidant, anti-cancerous and immunomodulating efficacy [69, 76]. These activities are summarized in Table 20.3.

Table 20.2 Microalgal activities as anti-cancer agents

Bioactive compound	Microalgal species	Activities	References
Scytonemin	*Cyanobacterium Stigonema sp.*	Anti-proliferative and anti-inflammatory effects (inhibits human fibroblasts and endothelial proliferation)	[70]
Curacin-A	*Curaco collection of Lyngbya majuscule*	Anti-proliferative effects (inhibits tubulin polymerization and shows inhibitory activity selectively on colon, renal and breast cancer-derived cell lines)	[71]
Largazole	*Symploca sp.*	Anti-proliferative activity	[72]
Aparatoxin A	*Lyngbya boulloni*	Cytotoxicity against adenocarcinoma cells	[73]
Coibamide A	*Leptolyngbya*	Cytotoxicity against NCIH460 lung and mouse neuro-2a cells	[74]
Borophycin	*Nostoc linckia and N. spongiaeforme var.tenue*	Cytotoxicity against human epidermoid carcinoma and human colorectal adenocarcinoma cell lines	[75]

20.4.5 Anti-inflammatory and Antinociceptive Properties

Inflammatory reaction is a characteristic for many diseases and conditions of health disturbance and diseases such as atherosclerosis [82], gastrointestinal dysfunction [83], lung diseases [84] and endothelial dysfunction [85]. It is well known that antioxidants exert anti-inflammatory effects, helping in alleviating or resolving the inflammatory condition [86–88]. Numerous marine algal bioactive components were found to have anti-inflammatory and antinociceptive properties. Table 20.4 presents the different biological activities obtained from various algal species.

20.4.6 Cosmetic Applications

The cosmetics industry is noticeably progressing on a global scope. Since July 2013, European Commission regulation No.1223/2009 defines cosmetics as "any material or mixture prepared to be applicable on the external parts of the human body such as epidermis, lips, nails or hair as well as on teeth or oral mucous membrane with the principle or exclusive purpose to perfume, clean, preservation or modification of their appearance. Marine algae provide a worthy source of bioactive ingredients for the cosmetic industry. Both macro- and micro-algae are rich in carbohydrates,

Table 20.3 Macroalgal activities as anti-cancer agents

Bioactive compound	Macroalgal species	Activities	References
Fucoidan	*Ascophyllum nodosum*	Anti-proliferative effects on both normal and malignant cells (including hamster kidney fibroblasts, sigmoid colon adenocarcinoma cells and smooth muscle cells) in addition to anti-tumor, anti-metastatic and fibrinolytic properties in mice	[77]
Stylopoldione	*Stypodium sp.*	Cytotoxicity (halts mitotic spindles formation)	[78]
Condriamide-A	*Chondria sp.*	Cytotoxicity against human nasopharyngeal and colorectal cancer cells	[79]
Caulerpenyne	*Caulerpa sp.*	Anti-cancer and anti-proliferative activities against human cell lines	[80]
Meroterpenes, and Usneoidone	*Cystophora sp.*	Anti-tumor properties	[80]
Sulfated polysaccharide	*Eclonia cava*	Suppression of cancer cell lines proliferation in vitro. Due to its high anticoagulant activity it demonstrated antiproliferative effect on murine colon carcinoma, human leukemic monocyte lymphoma, human promyelocytic leukemia and mouse melanoma cell lines	[81]

Table 20.4 Anti-inflammatory activities of marine algae [69]

Algal species	Algal type	Biological activities
G. verrucosa *G. textorii*	Red algae	Anti-inflammatory properties
G. tenuistipitata	Red algae	Anti-inflammatory properties against viral induced inflammation
Porphyridium sp.	Red algae	Inhibition of retroviruses replication
Polyopes affinins	Red algae	Suppression of asthmatic reaction
Neorhodomela aculeata	Red algae	Anti-inflammatory properties in neurological diseases: (1) inhibits cellular reactive oxygen species (ROS) generation; (2) inhibits H_2O_2 induced lipid peroxidation; and (3) inhibits inducible nitric oxide synthase
Delesseria sanguinea	Red algae	Anti-inflammatory properties
Bryothamnion triquetrum	Red algae	Anti-inflammatory and anti-nociceptive effects
Gracilaria caudate	Red algae	Anti-inflammatory and anti-nociceptive effects
Gelidium crinale	Red algae	Anti-inflammatory and anti-nociceptive effects
Hypnea cervicornis	Red algae	Anti-inflammatory and anti-nociceptive effects
Pterocladiella capillacea	Red algae	Anti-inflammatory and anti-nociceptive effects
Dunaliella bardawil	Green algae	Protection against acetic acid-induced small bowel inflammation due to its wealth with beta-carotene antioxidants
Ulva conglobate *U. lactuca*	Green algae	Anti-inflammatory effects
Chlorella marina	Green algae	Anti-inflammatory effects
Caulerpa Mexicana	Green algae	Anti-inflammatory and anti-nociceptive effects
Caulerpa cupressoides	Green algae	Anti-inflammatory and anti-nociceptive effects
Ecklonia cava	Brown algae	Reduced allergic airway reactions and inflammation
Ishige okamurae	Brown algae	Anti-inflammatory effects
Lobophora variegata	Brown algae	Anti-inflammatory and anti-nociceptive effects
Sargassum wightii	Brown algae	Anti-inflammatory effects on adjuvant induced arthritis in rats
Sargassum vulgare	Brown algae	Anti-inflammatory and anti-nociceptive effects
Spatoglossum schroederi	Brown algae	Anti-inflammatory and anti-nociceptive effects
Eisenia bicyclis	Brown algae	Anti-inflammatory effects
Ecklonia cava	Brown algae	Anti-inflammatory effects
Ecklonia kurome	Brown algae	Anti-inflammatory effects
Sagrassum siliquastrum	Brown algae	Anti-inflammatory effects
Spirulina platensis	Blue-green algae	Antioxidative effects and anti-inflammatory effects by inhibiting the production of pro-inflammatory cytokines and inhibiting inducible nitric oxide synthase and cyclooxygenase 2 expression

amino acids, proteins, vitamins (A, B and C) and minerals (iron, copper and zinc). Cosmetically, all those components are important for skin hydration, shine, firming and protection [89].

20.4.6.1 Skin Moisturizing Care

It is well known that skin integrity is preserved through the appropriate maintenance of hydration. This is commonly obtained through the application of certain provisions or formulations intended to limit water loss such as lipids. Several different moisturizing components are obtainable from marine algae such as proteins, polysaccharides and fatty acids [89]. In general, restoring trans-epidermal water loss to the appropriate levels is related to omega-6 polyunsaturated fatty acid particularly linoleic and linolenic acids [90]. Remarkably, high linolenic acid content is obtained from Nannochloropsis micro algae [89]. In addition, macro algae are wealthy in serine, such as *Undaria pinnatifida* which is of particular cosmetic importance [89, 90].

20.4.6.2 Skin Anti-aging

Obviously, skin aging is strictly associated with the degenerative processes of epidermal and/or dermal extracellular matrix. Extracellular matrix principally consists of hyaluronic acid and syndecan-4 protein. Thus, skin anti-aging care is largely dependent on synthesis and induction of both of these components. An aqueous extract of the brown alga *Macrocystis pyrifera*, which belongs to the family Laminariaceae, is widely applied in skin care products and is obtainable from markets [91]. Furthermore, carotenoids, which are liposoluble pigments, serve as principle anti-aging ingredients. Carotene, which leads the family of carotenoids, is very capable of forbidding the formation of reactive oxygen species (ROS) as well as acting as provitamin A. This pigment is mainly produced by the halotolerant microalga *Dunaliella salina* that is capable of producing 10% carotene in comparison to its dry weight [92].

20.4.6.3 Skin Photoprotection

As photosynthetic organisms, marine algae provide important resources for photoprotective compounds especially ultra-violet (UV)-absorbing agents like: scytonemins, mycosporines, mycosporine-like amino acids (MAAs) and carotenoids [93, 94]. MAAs are capable of absorbing UV radiation with wavelengths from 310 to 362 nm and disperse this energy in the form of heat radiation to the peripheral environment [95, 96]. The protective capacity of MAAs against UV relies on the location of these ingredients within the cell. The cytoplasmic MAAs shows a limited protective efficacy in comparison with the extracellular ones that are more protective [95].

Scytonemin is an extracellular matrix constitutive pigment that is found in some cyanobacteria species. It is an excellent absorbent of both UVA and UVB radiations. It is also capable of reducing up to 90% of UVA radiation into the cells, thus it is widely applied in the sunscreen products to prevent skin damage [95, 96].

20.4.6.4 Skin Whitening Compounds

There is an increasing demand on skin whitening cosmetics for different medical and esthetic purposes such as hyperpigmentation, lentigo, pregnancy mask and many other reasons. It is well known that tyrosinase is the principle enzyme for melanin synthesis. Anti-tyrosinase activity is the key principle of many whitening products. Tyrosinase inhibitors are very obtainable from marine organisms, with *Clorella* extract demonstrating reduction of skin pigmentation by more than 10%. Moreover, 7-phloroeckol which is a phlorotannin derived from *E. cava* brown seaweed as well as Zeaxanthin derived from *Nannochloropsis oculata* are of particular interest as skin whitening agents [97].

20.4.7 Food Industry

At the present time, algal biomass represents an alternative nutritive resource for the world's population. Marine algae are rich in different nutritive components such as polysaccharides, proteins, lipids, vitamins, minerals as well as dietary fibers. All of these aforementioned components are significant not only as an alternative food but also in improving health conditions and solving the problem of malnutrition [98]. In the coastal areas, marine algae are vastly cultivated and provided as food for both humans and animals. Species that showed the greatest interest to consumers as described by the food industry are mainly those from *Porphyra* sp., *Chondrus crispus, Himanthalia elongata* and *Undaria pinnatifida.* The latter is attributed to their low calories content and rich content of vitamins, minerals and dietetic fibre [99], even though microalgal biomass also provides significant nutritive ingredients. They are generally provided in the form of powders, liquids, tablets or capsules, which can be incorporated into various foods. On the other hand, the microalgal consumption is restricted to very few taxa, which mainly include *Spirulina* and *Chlorella* genera. *Spirulina* is the wealthiest and the most complete origin of organic nutrition; additionally, it has various capabilities of health promotion and enhancement. Moreover, Chlorella has been used as traditional food and in current food markets as long as it is rich in significant health promoting factor concerning many kinds of disorders [99].

20.5 Tissue Engineering and Regenerative Medicine Applications

As soon as the human body experiences a trauma, injury, or disease to one of its organs, a very complex cascade of physiological and biochemical events occurs in order to remodel the injured portion; however, not all the human tissues have the ability to successfully undergo these manifold occurrences. Accordingly, the natural healing process sometimes results in dysfunctionality and subsequently tissue repair failure [100].

To overcome such severe health issue, some technique such as prosthesis, autografts and allografts have been developed during the past decades and the results obtained certainly represented a significate step forward in the clinical treatment of such injured tissues. However, they are still very restrictive due to a number of drawbacks such as immune reactions, infections, site morbidity, donor scarcity and inappropriate mechanical and morphological capacity. Due to these important and unanswered questions, Langer and Vacanti introduced the possibility of combining the efforts from multidisciplinary approaches such as engineering, biology and life sciences with the ambitious aim of "*biofabricating*" new tissues using cells and biocompatible materials [101]. Through the introduction of this visionary concept of replacing tissues rather than improving their self-healing ability, the "Regenerative Medicine" era was initiated.

At the base of the regenerative medicine concept, a porous material referred to as scaffold with defined mechanical and physiological properties is designed and produced in order to host cells adhesion and proliferation thus acting as a functional tissue substitute ready to be implanted in the injured site. As a logical consequence, the scaffold properties must be optimized to (i) improve the interaction with cells and (ii) to mimic the three-dimensional features (intended as porosity, topography and interconnectivity) of the naïve tissue where it will be implanted. Therefore, to create the optimal scaffold shape, different technologies have been applied. Some of these technologies include foaming [102], 3D printing [103], particles aggregation [104] electrospinning [105], supercritical fluids [106] and fibers bonding [107].

Just as the choice of the processing technique is of pivotal importance to obtain the desired scaffold, the selection of the basic material represents a crucial step in the success of the entire process. From this point of view, naturally derived as well as polymeric materials have been proposed as a stand-alone material or combined with other materials for the manufacturing of biocompatible scaffolds. Amongst the wide selections of candidates, glycosaminoglycans have raised a great attention due to their ability to influence progenitor cells adhesion and subsequent differentiation by regulating gene expression [108, 109]. In particular, hyaluronic acid and chondroitin sulfate have been identified as very promising candidates owing to their immunogenicity and their affinity towards certain growth factors that must be recruited to ensure the healing process. Interesting, studies have demonstrated that glycosaminoglycans are able to maintain these peculiarities not only in a physiological environment, but also in pathological processes [109]. In particular, the degree

of sulfation seems to be a key factor in promoting regeneration: when sulfate features were introduced into hyaluronic acid, the ability to bind growth factors was significantly increased thus determining a stronger and faster activation of seeded cells [110]. However, these results seem to be related with the type of assayed cells as some literature evidenced an improving in dermal fibroblasts proliferation [108] while some other authors observed a marked hindering for osteoblasts [111].

A more noticeable effect of sulfate-rich glycosaminoglycans can be observed when the fates of stem cells are manipulated. In fact, both bone marrow and adipose tissue-derived mesenchymal stem cells were stimulated to produce osteoblast-like type I collagen by matrices composed by sulfate hyaluronic acid [112, 113]. As a further demonstration, the use of hyaluronic acid-free self-assembled ϖ-sulfatealkanethiols monolayers as scaffold for stem cells was sufficient to promote osteogenesis as well as a high cells affinity as demonstrated by the formation of numerous filopodia [112].

20.5.1 Marine Algae as Source of Polysaccharides

As discussed previously, once the capability of glycosaminoglycans sulfate groups in determining the outcomes of stem cells in regenerative medicine was established, investigations have begun by the research community to establish where stem cells can be sourced and how they can be acquired. A very important answer to this need was obtained from marine algae where they serve as an abundant source of natural polysaccharides as well as an underestimated supply of biocompatible biomaterials [114, 115]. Moreover, the use of these plants-derived polysaccharides seems to be a safer option in comparison to the mammalian alternatives because their associated risk of causing severe diseases when applied in human patients is not considered a real risk [115]. Accordingly, the most representative sulfate polysaccharides, which may be obtained from marine algae (red and brown) and their potential application for tissue engineering and regenerative medicine purposes, are discussed below.

20.5.1.1 Red Algae-Derived Polysaccharides

The marine red algae *Rhodophyceae*, such as *Chondrus crispus, Eucheuma cottonii and spinosum*, are rich sources of carrageenans sulfated polysaccharides; those hydrophilic colloids are highly sulfated galactans, with sulfate content ranging between 15 and 40% with molecular weight ranging from 10^5 to 10^6 Da [116]. Their primary structure is based on repetition of β (1-4) and α (1-3) linked D-galactose residues. However, due to the different environmental origin of the algae, the sulfate units can vary within the galactose units permitting the possibility of identifying between kappa, iota and lambda families.

For tissue engineering purposes, the interest towards carrageenans is related to the possibility to obtain: (i) hydrogels (with the exception of lambda family) and; (ii) microscale fibers (by combining different families).

Hydrogels (named alginate) can be easily obtained due to the high hydration capacity of carrageenans despite the fact that the entire chemical process is still unknown. Moreover, these hydrogels may be synthesized using ionic gelation through the introduction of cations such as potassium and they can display thermoreversible ability to move from gelation to solution phase simply by applying an external stimulus such as temperature or pH [117]. Another interesting feature of carrageenans hydrogel is characterized by their thixotropy, intended as the ability of spontaneously regaining viscosity after mechanical disruption [118].

In addition to hydrogels, carrageenans can also be utilized to obtain fibers by wet spinning, which involves immersion into a cross-linking solution or into porous structures by freeze-drying [119]. Furthermore, by combining the two approaches, it is possible to produce microscale fibers [120]. Carrageenans hydrogels can be also manufactured as nanoparticles to be combined with other materials such as chitosan in order to obtain nano-delivery systems [121]. Moreover, they can be used in combination with nanotubes to improve hydrogel mechanical properties for orthopedic applications [122].

The high reactivity of carrageenans sulfate groups make them interesting chemicals due to their ability to react easily with charged proteins. For biomedical purposes, their strong antioxidant activity [123] as well as their antibacterial properties has been documented [124]. Once applied to a clinical trial, carrageenans considerably decrease serum cholesterol and triglyceride levels [125], showing also anticoagulant properties [126].

20.5.1.2 Brown Algae Derived Polysaccharides

Brown marine algae are in general very rich in polysaccharides such as alginic acids (alginate) or laminarins (laminarans); however, the most interesting one is represented by sulfated fucans, namely fucoidan, which is considered as potential therapeutic agents [127]. Fucoidan represents 5–20% of the algae dry weight and it was described for the first time as a potential active principle in the 70s [128]. Molecular weight of fucoidan ranges from 13 to 950 kDa while two main families have been identified based on the chemical structure: one group includes *Laminaria* species that have their central chains composed by (1-3)-linked a-L-fucopyranose residues while a second group includes fucoidan isolated from *Ascophyllum* and *Fucus* species that have their central chains composed of repeating (1-3) and (1-4) linked α-L-fucopyranose residues.

Unlike carrageenans, fucoidan may only be capable of forming a viscous solution and not a gel, therefore this prevents the possibility of fucoidan to produce hydrogels [129]. However, due to its high bioactivity, fucoidan has been shown to own antitumoral [129], anti-coagulant [130], anti-viral [131], and anti-inflammatory activities [129]. Accordingly, to exploit the strong bioactivity of fucoidan, it has been combined

Table 20.5 Biomedical application of marine polysaccharides-based biomaterials (Modified from [135])

Polysaccharide	Origin	Application
Agarose	Red algae	Antibacterial
Alginate	Brown algae	Antifungal; anticancer
Carrageenan	Red algae	Antioxidant
Fucoidan	Brown algae	Anticancer
Ulvan	Green algae	Wound dressing

with other material for tissue engineering purposes. For instance, fucoidan can act as an anti-coagulating agent when it is cross-linked with chitosan [132, 133], while PCL electrospinned fibers have been used as carrier for fucoidan to induce pro-osteointegrative properties in bone regeneration [134].

20.5.2 Biomaterials Applications for Tissue Engineering

As discussed previously, marine algae have attracted a lot of attention as a natural source of marine natural products as a consequence of their popularity as a choice for sulfated polysaccharides. Accordingly, their applications in biomaterials science are rapidly increasing due to the possibility to exploit algae derivates as scaffold or as active principles in combination with other biocompatible materials. Some examples of algae derivates biomaterials are summarized in Table 20.5.

Biomaterials derived from marine algae polysaccharides can be manufactured using different techniques based on their intended application. Some of the most promising approaches along with their regenerative potency are described below.

20.5.2.1 Nanoparticles

Marine polysaccharide-based nanomaterials have drawn significant and numerous supports from nanoscience and nanotechnology scholars recently. This is due to their excellent and promising biocompatibility, high biodegradability, low cost, and non-toxic nature. Furthermore, they have been utilized as novel carriers for imaging and therapeutic agents due to their unique physicochemical characteristics. Consequently, marine polysaccharide-based nanomaterials will play significant role into several fields of life science in the not so distance future.

Fucoidan (described in Sect. 20.5.1.2) has been employed as coating for poly(isobutylcyanoacrylate) nanoparticles exhibiting targeted toxicity towards J774 macrophage and NIH-3T3 fibroblast cell lines [136]. Fucoidan also demonstrated a strong anticancer activity towards osteosarcoma cells when used as reducing agent for the biosynthesis of silver nanoparticles [137]. The same strong anticancer activity

was observed by combining fucoidan with doxorubicin during the manufacture of acetylated fucoidan nanoparticles [138].

Alginate is a natural polysaccharide obtained from the brown algae; it is, at the moment one, of the most utilized natural materials in drug delivery system due to its outstanding and exceptional biocompatibility, low toxicity, low cost, and mild gelation. Alginate nanoparticles play an important role in the delivery of insulin. Alginate by itself or combined with dextran represents a very efficient vehicle for insulin with an encapsulation efficiency of 82.5% [139]. In studies related to anti-cancer treatments, doxorubicin-loaded glycyrrhetinic acid-modified alginate nanoparticles were utilized for liver malignant tumor chemotherapy like the hepatocellular carcinoma; nanoparticles demonstrated powerful liver-targeting efficiency owing to both the passive targeting through the enhanced permeability and retention effects and to the glycyrrhetinic acid active targeting efficiency [140].

Another important application of alginate nanoparticles is the targeted treatment of tuberculosis. In fact, the therapeutic disability of tuberculosis is predominately due to patient non-compliance, for example diagnosed to require multidrug administration daily or several times a week. Alginate-encapsulated antitubercular drugs showed to be very effective in drug encapsulation (80–90% yield) and to be able to deliver and maintain drugs level at a much longer and higher rate than mice receiving free drugs at equivalent doses [141].

Carrageenan (described in Sect. 20.5.1.1) is a naturally occurring biopolymer acquired from marine red algae. It can be used to obtain magnetite nanoparticles via biosynthesis technique [142] or in combination with chitosan to obtain inexpensive and cytocompatible nanoparticles by hydrophilic status utilizing very moderate steps and excluding the application of organic solvents and other aggressive conditions [143]. Furthermore, the possibility of obtaining thermo-reversible hydrogels from carrageenan allows the manufacture of controlled drug delivery systems containing gold rods and nanoparticles where they were successfully delivered to the gastrointestinal tract with a higher efficiency and in a controlled manner than free administration [144].

Agarose is a linear biopolymer composed of repeating units of agarobiose that is a disaccharide made up of d-galactose and 3-6-anhydro-l-galactopyranose. It is normally employed in molecular biology and biotechnology arenas for the isolation of biomolecules specifically DNA by electrophoresis. Owing to this high affinity for DNA, agarose nanoparticles have been successfully used to detect micromolar concentrations of DNA nucleosides using surface-enhanced Raman spectroscopy [145].

20.5.2.2 Nanofibers

Ulvan is a complex anionic sulfated polysaccharide isolated from the cell walls of marine green algae (*Ulvales*, *Chlorophyta*). The major components of ulvan are sulfated, xylose, rhamnose, glucuronic, and iduronic acids. Ulvan is a promising choice for nanofiber synthesis, which has been successfully introduced into nanobiotech-

nology. Currently, it is mainly utilized for the preparation of nanofibers that can be applied for drug delivery, wound dressing, and tissue engineering. Above all, ulvan-derived polysaccharides can be easily manufactured into nanofibers with great stability and mechanical properties using common electrospinning technique [146]. At the moment, these nano-layers shows great promise in a number of tissue engineering applications.

20.5.2.3 Biomedical Applications

Due to the large number and usages of marine algae derivates, a large number of biomedical applications have been reported in the literature.

Antimicrobial characteristics have been shown for different compounds extracted from marine algae. Silver nanocomposite material extracted from the red alga *Gracilaria dura* demonstrated greater bactericidal effect, with 99.9% reduction in bacteria compared to control samples [147]. Similar results were displayed by silver nanoparticles isolated from marine red algae *P. vietnamensis* that were highly effective towards Gram-negative strains [148].

The possibility of utilizing nanobiotechnology in medicine to create a more efficient drug delivery as well as in the pharmaceutical industry is set to spread rapidly. However, the fact that the delivery system itself represents a source of risk has been greatly underestimated due to the possible toxicity arising from the selection of materials used. From this point of view, the choice of natural-derived materials from marine algae represents a very attractive alternative due to the risk of these materials of introducing toxic elements is very low [149].

Gene therapy has recently become a potential strategy to treat diseases. From a genetic point of view, materials with high affinity to nucleic acid are highly desirable. Alginate and chitosan-alginate materials displayed the highest affinity towards DNA, even in a very small amount, making them a very useful and promising tool for gene therapy. In particular, the coupling of DNA or RNA with alginate nanoparticles revealed that nucleic acids were protected from degradation due to nuclease attack thus resulting in a very efficient delivery [150].

In cancer treatment, one of the most important issue is related to the high wide-spectrum toxicity of chemotherapeutic agents. Therefore, an efficient and targeted delivery system could represent an important step forward: attributed to the existence of carboxymethyl groups, marine algae polysaccharides can efficiently bind drugs such as doxorubicin thanks to the link with its amine groups. This complex was able to improve the activity of doxorubicin in a relevant manner while, at the same time, reducing the amount of toxicity in comparison with the free-drug administration [151].

Lastly, nanofibers obtained from electrospun marine algae polysaccharides can be used as biosensors due to their high stability; for instance, a new electrochemical tyrosinase biosensor for determining phenolic compounds was developed based on the application of a glassy carbon electrode modified with tyrosinase–Fe_3O_4

magnetic nanoparticles and chitosan was successfully used to detect phenolic contaminants in environmental samples [152].

20.5.3 Threats to the Industrial Applications of Micro- and Macro-algae

The Egyptian coastline extends over a length of 3000 km of which nearly 1150 km extend along the Mediterranean, from Salloum west to Rafah east, and 1850 km covering Egyptian Red Sea coastline on the main Red Sea basin (some 1200 km) and both, Suez and Aqaba Gulfs (approximately 650 km). Both Egyptian marine environment and coastal zones are regarded as a renewable origin for living and non-living wealth and a pivotal point of attraction for several projects in a verity of economic and social fields like recreational and tourist projects and others related to fish wealth, manufacturing and international trade projects which are mostly affected by several terrestrial and marine sources of pollution [153].

As reported by the UN Reports [154] and finally confirmed by several field studies and research investigations, almost 80% of marine environment pollution and coastal deterioration are assigned to multiple land activities such as agricultural, industrial, urban or physical wastes and emissions. The remaining 20% is attributed to different marine sources: different activities like oil, mineral and natural gas investigation and drilling processes, along with shipping, fishing, and uploading and marine transport. Moreover, other sources such as wastes and leakages due to the increasing number of ships, yachts and other recreational and tourist boats currently present. The aforementioned activities, if not environmentally rationalized, will have unfavourable effect on marine environment and wealth and on different faces of development and investment activities. They also impose many threats to human health as well [153].

The study of the contaminant heavy metals in the coastal sediments of El-Hamrawein seaport over the Egyptian Red Sea coastal line is an issue of considerable urgency due to the powerful impact of phosphate shipment operations alongside the derived materials from the close by wadis (e.g., Wadi Hamrawein), on the marine environment in this region and its surroundings. Massive clouds of dust result from phosphate loading procedures at El-Hamrawein harbor, that ultimately subside over the relative seacoast or immediately falling into the water depending on the wind trend. As a result, a deviation in the environmental stability is the result of the abnormal increase in the nutrient content from this dust. Huge quantities of metals are contained within the rock phosphates particularly cadmium and zinc as impurities [155]. The environmental hazard caused by metals relies largely on their forms in water, the metal amplitude for complexing, precipitation and bioaccumulation [156, 157]. In marine ecology, precipitations are implicated as a substantial tool for assessment of heavy metals pollution, due to their long-lasting lodging time. Several investigations have considered the recent precipitants and human influence on the Egyptian Red Sea coast [157–167], but investigations regarding the influence of

developmental trends along the Red Sea coast are still few [168–170]. All of such activities and pollution sources influence the nature of marine plant or marine algae in the involved area.

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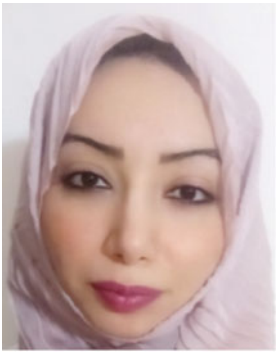

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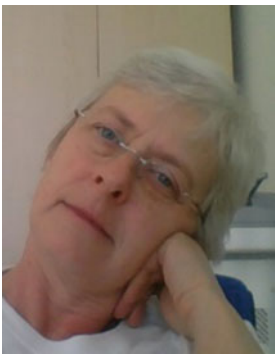

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Chapter 21
Chitin Nanomaterials and Nanocomposites for Tissue Repair

Pierfrancesco Morganti, Gianluca Morganti and Maria Beatrice Coltelli

Abstract Chitin nanomaterials and nanocomposites are being used more frequently in tissue repair and skin rejuvenation. In fact, with the increase in age, the prevention of skin diseases became a necessity for our society together with an amelioration of the general body appearance. Thus, while medications have been revolutionized in their structure by the use of biodegradable and nature-oriented non-woven tissues, the use of natural polysaccharides polymers in cosmetics such as beauty masks are becoming increasingly manufactured and sold to the public. This chapter reports the biological properties and applications of biopolymers as well as discussing the physicochemical characteristics and the skin repairing activity of non-woven tissues based on the use of chitin nanofibrils obtained from biomass.

Keywords Chitin nanofibril · Lignin · Silver · Skin repair · Extra cellular matrix · Nanocomposite · Nanomaterial · Nanofiber · Smart carrier

21.1 Introduction

According to a report by the United Nations (UN) [1], the worldwide population is expected to reach 9.8 billion in 2050 and 11.2 billion in 2100 with an increase in life

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expectancy (Fig. 21.1). Consequently, in 2050, the aged population is estimated to be around 1.6 billion in developing countries, thus resulting in enormous socioeconomic and political impacts. The aging process at a rapid rate and with the baby boomer generation at the geriatric doorstep will continue to set the trend for the next 30 years [2].

Moreover, an increase in the longevity of humans and their dynamic migration into different parts of the world will result in an increased demand on the health sector with questions to health-care provisions and costs as well as the health needs of the elderly and the migrating populations [2]. As a consequence of this normal aging process and migration, the increase in life-long exposure to environmental and chemical agents that results in modifications in the natural microbiome, is the primary reason behind the worldwide increase in chronic diseases. These phenomena which affect the elderly, contributes to their disability and reduces their quality of life [3]. Additionally, in our current society and in an age where we are greatly influenced by the environment and social media, there is also a growing concern about our body appearance. Understanding and mapping out the interactions between human beings and their environment is therefore a huge task, like the mapping of human and non-human microbiome. For these reasons, the effects of UV and IR radiation, tobacco smoke, ozone, pollutions caused by nanoparticles inside and outside our homes have also received an increasing amount of attention (Fig. 21.2). Through the combined efforts of innovative and challenging research studies, we are discovering ways to slow down premature skin aging and diseases. These phenomena have created a further challenge for the healthcare system and society, which include major investments in advanced medications to ensure innovation, quality, safety, and value in healthcare [4].

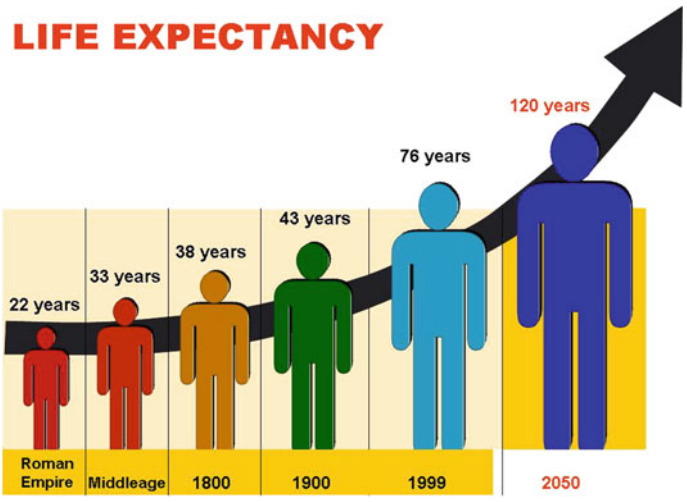

Fig. 21.1 The increase in life expectancy of the population worldwide by 2050

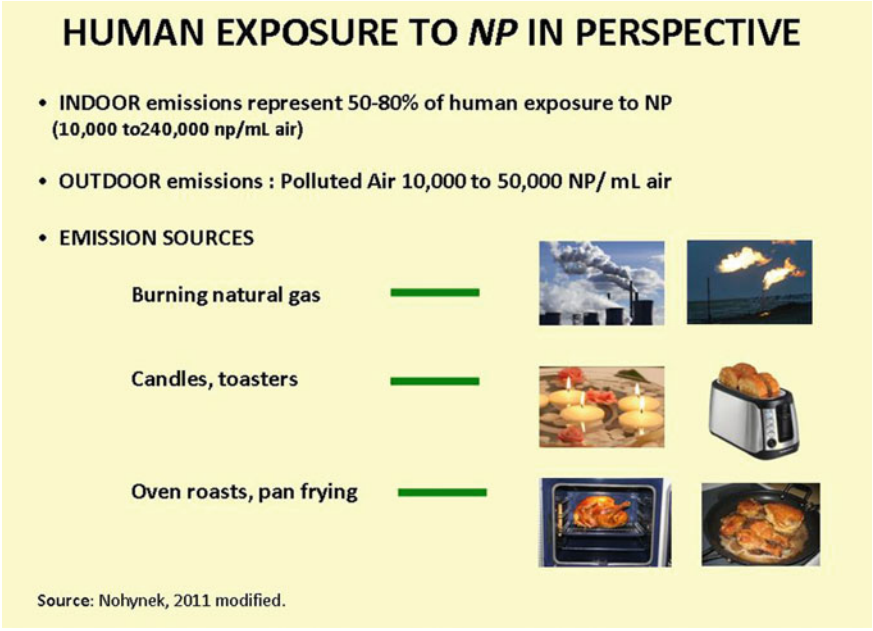

Fig. 21.2 Pollutions related to the use nanoparticles inside and outside our homes

The need to develop innovative strategies related to for instance the prevention of skin diseases and skin cancers in our aging society involves numerous innovative and original ideas from all medical disciplines. Therefore, the development of nanotechnology-based tissue engineered novel advanced dressings and tools could assist in the skin wound healing processes and/or bone reconstruction in difficult cases. Subsequently, tissue engineering, which is based on the use of porous biodegradable scaffolds, can provide temporary support for cell growth, as these scaffolds displays the same structure as the natural extracellular matrix (ECM) (Fig. 21.3). Such scaffolds may be produced by agglomeration of micro/nanospheres and/or micro/nanofibers made of biocompatible materials arranged into a three-dimensional (3D) structure, such as the non-woven tissues (Fig. 21.4) [5].

More importantly, these structures should stimulate the production of protein factors necessary for the injured bone or chronic skin wounds to heal and permitting the proper growth of new tissues. For all these reasons, the global nanotechnology-based market in advanced medications (Fig. 21.5) is expected to grow by 11–12% during the forecast period between 2012 and 2022, driven by the growth in the aging population worldwide, as well as our increased use in biomedical devices and tools, and the increase in research and development investments [6].

These medications must be innovative in their structure and composition and can be fabricated using non-woven tissues or films made by natural and biodegradable polymeric fibers, possibly obtained from agricultural and industrial by-products. The

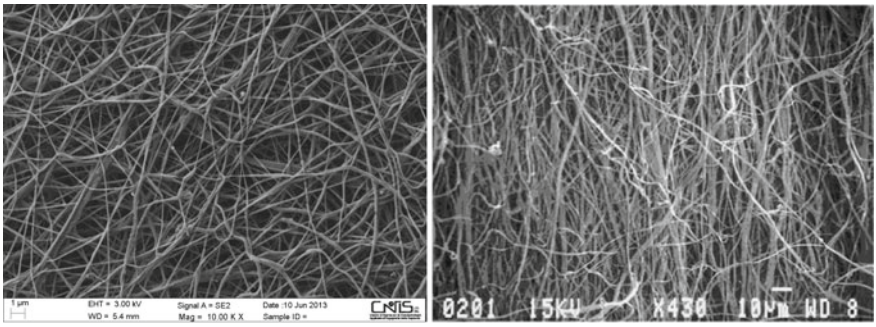

Fig. 21.3 SEM images of Chitin nanofibril structure (left) and native extracellular matrix (right)

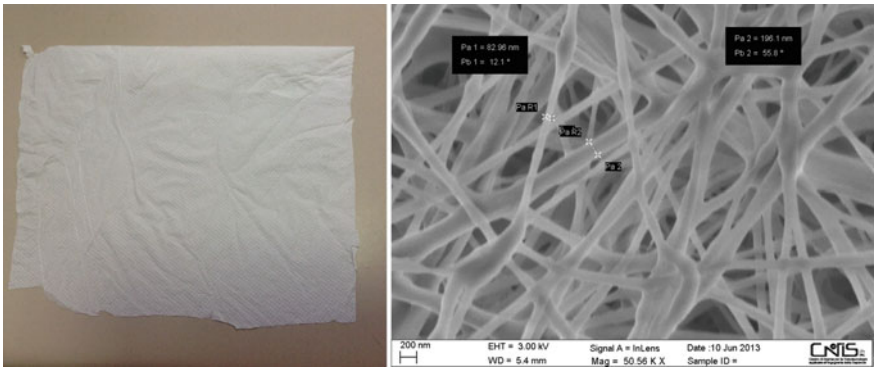

Fig. 21.4 Chitin nanofibril non-woven tissue (left) and SEM image (right)

reason in using these environmental-friendly materials and processes is due to the increasing concerns regarding global warming, carbon emissions, ozone layer depletion, environmental hazards and the need to reduce waste [6, 7]. These technological advances, in fact, are set out to replace finite resources and conventional industrial processes with bio-components, obtained from renewable resources, and bio-based processes, more sustainable and emitting less CO_2.

For this purpose, natural polymers are renewable and cheap compounds which pose minimal health hazards and can provide a solution to environmental pollution by finding new uses for waste materials [7]. Among these polymers, the most promising are polysaccharides, such as chitin and chitin-derived compounds, because of their outstanding biocompatibility and biodegradable properties, making them useful in the production of nanocomposites and scaffolds for medical applications.

Fig. 21.5 Expected spending on advanced medications in North America until 2022

21.2 Chitin Nanofibrils

Chitin, as the second most abundant biopolymer in nature after cellulose, is an unbranched glucose-derived compound comprising repeating monomer chains of N-acetyl-D-glucosamine [8]. The alpha form of this biopolymer, widely distributed as supporting structure in the cell wall of fungi and exoskeleton of arthropod and insects, is organized by amorphous and crystalline chains, arranged in various structural levels (Fig. 21.6) [9]. It is organized in an antiparallel fashion with highly ordered orthorhombic symmetry to form nanofibrils assembled into a honeycomb shaped array [10], similar to those observed in many human tissues (Fig. 21.7).

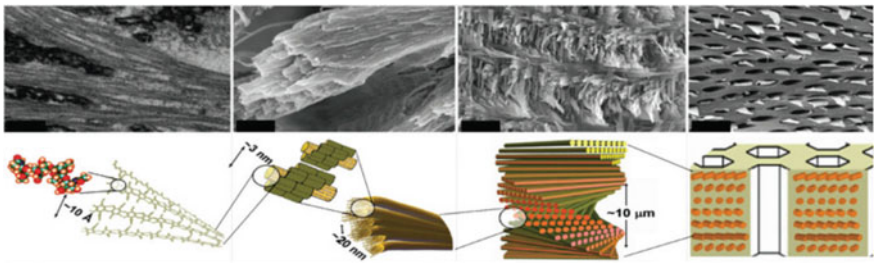

Fig. 21.6 Chitin nanofibrils assembled into a honeycomb shaped array (By courtesy of Nikolov et al. [9])

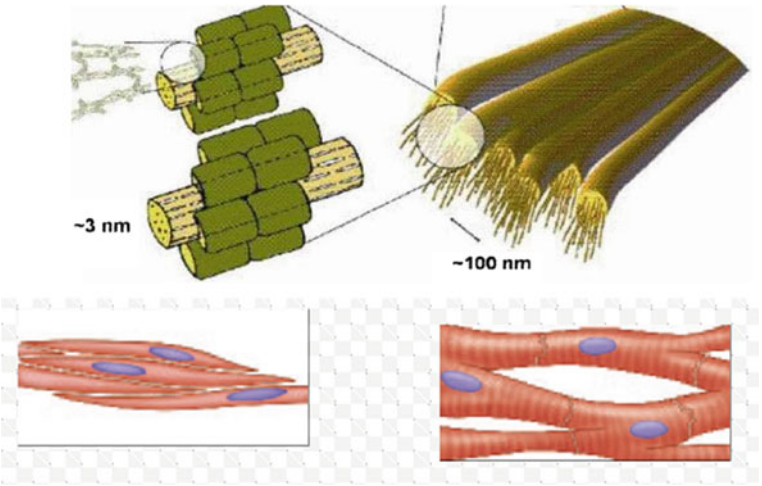

Fig. 21.7 Chitin fibrils are organized in crustaceans by a similar structure recovered in human smooth/plain muscle (down left) and in cardiac muscle (down right)

According to Tishchenko et al. [11], the shape of chitin nanofibrils suspended in water, both in the swollen state (Fig. 21.8a, b) and in the dry state (Fig. 21.8c, d) are quite similar. These nanofibrils with a needle-like appearance are slightly longer and thinner and with mean dimensions of 240 × 7 × 5 nm (shown in Fig. 21.9). Moreover, due to their cationic characteristics, the needle-like crystallites remain suspended in water and spontaneously reorganizing their chiral nematic structures with the water molecules [12]. Their x-ray diffraction pattern has an appearance of a well-ordered crystalline phase with peaks characteristic of alpha chitin (Fig. 21.10) [11].

Interestingly, it is important to underline how copper and silver nanostructured ions and utilized at their nanodimension, can bind on the surfaces of chitin nanofibrils without modifying their needle aspect [11], and at the same time enhances their antimicrobial activity (Fig. 21.11) [13]. In addition, it is important to describe that silver nanoparticles display the most significant antibacterial and antifungal properties and lower side-effects, which are different to the bulk silver metal [13, 14].

21.2.1 Biological Properties and Applications

Due to their specific characteristics of physical cross linkers, these pure and crystalline nanofibrils, being positively charged, could be separated from each other through certain technologies and used as fillers to strengthen the fiber structure of biopolymers or to make bio-based polymeric nanocomposites, as well as to produce innovative delivery carriers entrapping micro/nanoparticles, electro-negatively

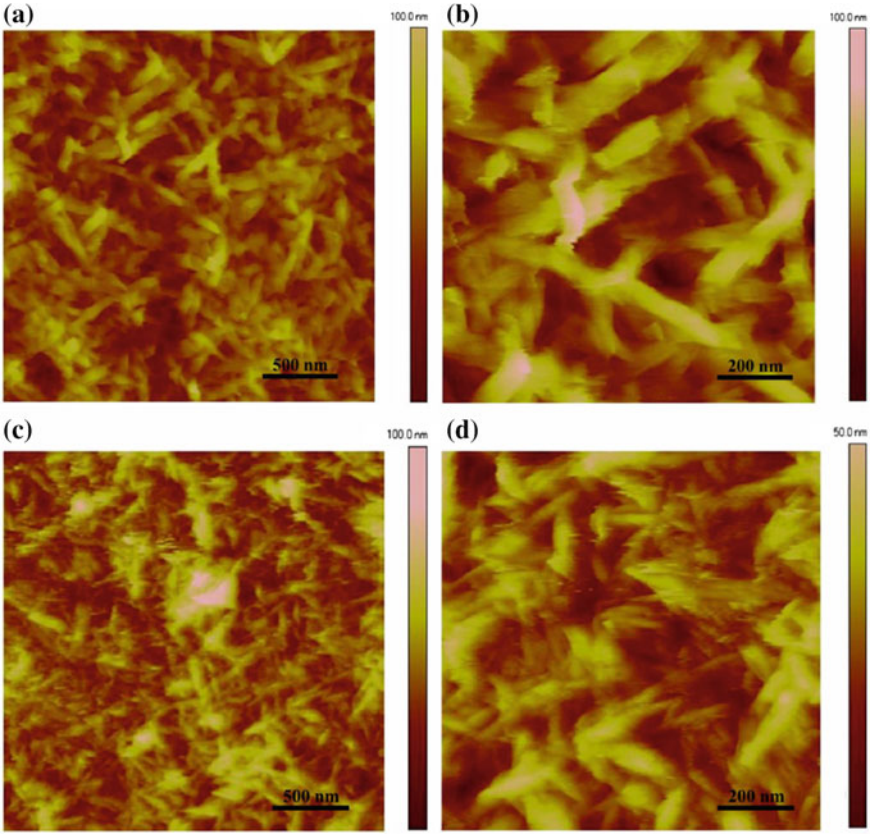

Fig. 21.8 AFM topography images of chitin nanofibrils in the swollen state (**a** and **b**) and in dry state (**c** and **d**). (By courtesy of Tischenko et al. [11])

or positively charged on their surface [13, 15, 16]. These charged nanoparticles, utilized as such or entrapped into emulsions or tissues, could utilize the benefits of electrostatic attractions with cellular membranes to enhance adsorption and subsequent cellular internalization. However, the charges have different effects on skin permeation. For instance, positively charged nanoparticles are more inclined towards contact/adsorptions on the cell membranes, largely consisting of negatively charged domains, such as phospholipids with negatively charged head groups and polysulphated proteoglycans and carbohydrates found in mammalian cells [13, 15]. On the contrary, negatively charged particles will remain on the skin, covering its surface as a film.

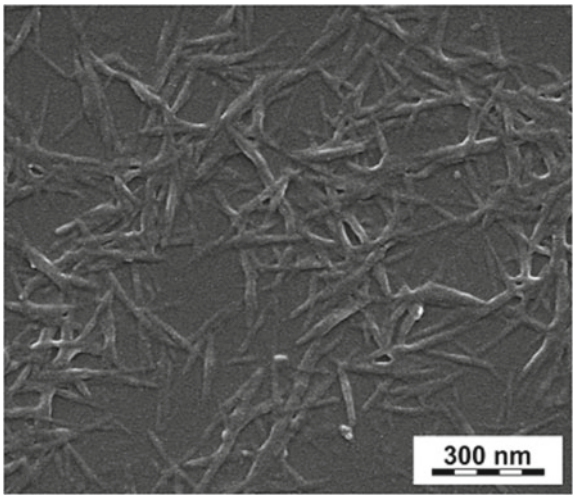

Fig. 21.9 TEM image of chitin nanofibrils

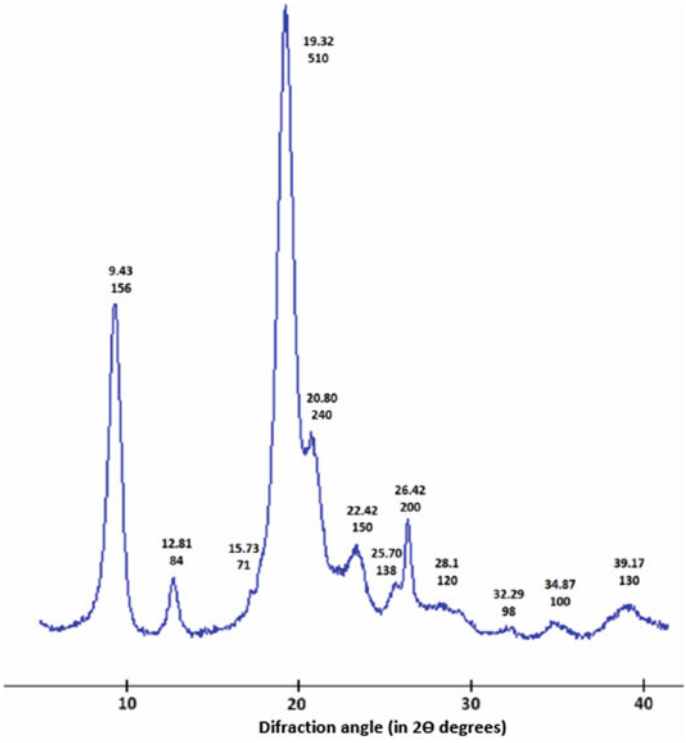

Fig. 21.10 X-ray diffraction pattern of alpha-chitin nanofibrils. (By courtesy of Tischenko et al. [11])

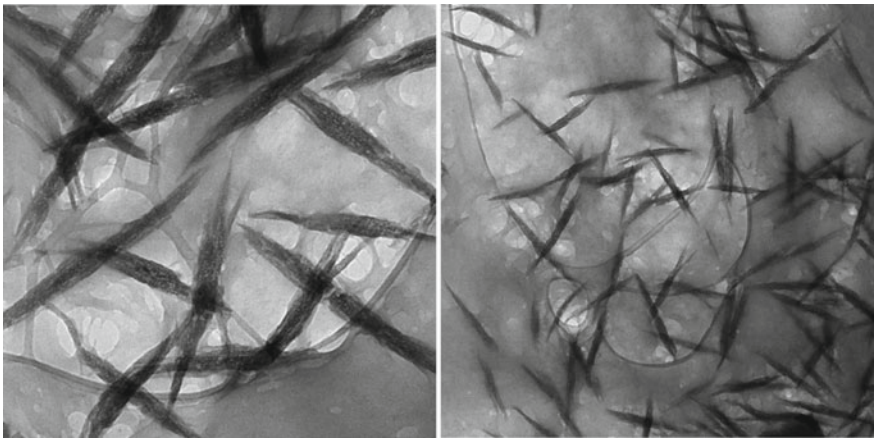

Fig. 21.11 TEM images of chitin nanofibrils bound to copper nanostructured ions

21.2.2 Production Methods

With respect to their industrial production, it is important to mention that these bio-carriers can be produced simply by combining electro-positively and negatively charged micro/nanoparticles in water solution using the ionic gelation method (Fig. 21.12) [17–19]. Using this approach, based on the ability of polyelectrolytes to cross-link in the presence of counterions, it is possible to form hydrogel beads called nanocapsules or gelispheres (Fig. 21.13) [18, 20]. If necessary, this technique can be easily adjusted to achieve desired polymer/surface modifications so that hydrophobic and hydrophilic ingredients can be simultaneously encapsulated [17, 20]. These gelispheres is also capable of entrapping different active ingredients and, based on the type and concentration of electrolytes used, could be designed as delivery systems to ensure stability and a constant time-release rate of the entrapped ingredients [13]. According to the so-called 4R's by Wiechers [21], "the role of a delivery system is to ensure that the *right* concentration of the *right* chemical is reaching the *right* site in the body for a *suitable* period of time".

21.2.3 Biopolymers Versus Bio-Based Polymers

Fundamental characteristic of both biopolymers and bio-based polymers must be their nanoscale dimensions, biodegradability and biocompatibility [19]. Thus, while biopolymers produced by living organisms are always biodegradable, the bio-based ones, derived from renewable resources, may not necessarily be biodegradable [22]. One such example is bio-polyethylene which is obtained from biomass through polymerization of ethylene synthesized from ethanol production.

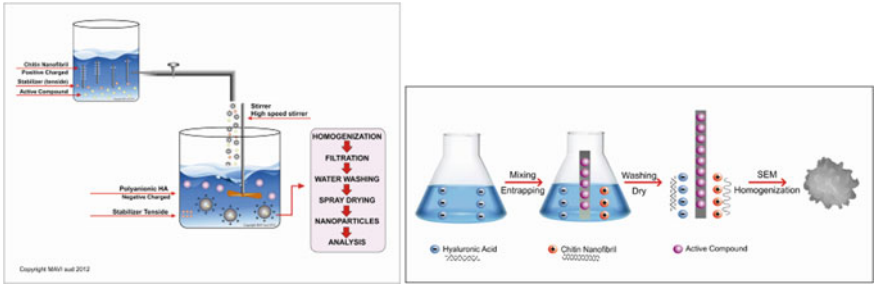

Fig. 21.12 Ionic gelation method used in the production of nanoparticle's complexes formulated, for example, between chitin nanofibrils (electropositive) and hyaluronic acid (electronegative)

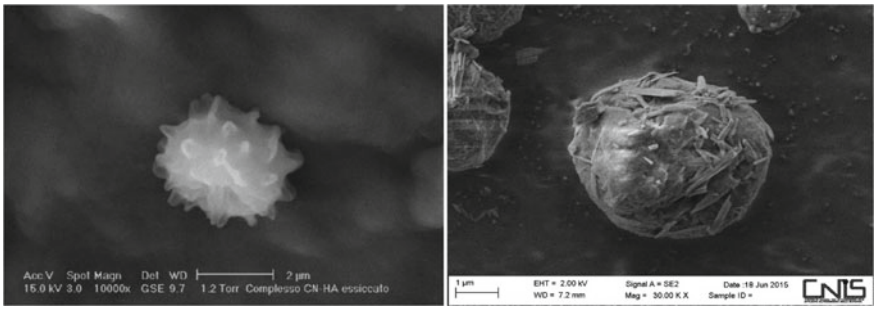

Fig. 21.13 Examples of cross-linked chitin nanofibrils-hyaluronan (left) and chitin nanofibrils-lignin (right) nanocapsules

Nanocomposites, on the other hand, demonstrate significantly improved material properties in comparison to pure polymers or conventional composites. Their performances are in fact dependent on the nanoparticles used and its features such as dimensions, aspect, specific surface area-to-volume ratio, compatibility with the matrix, dispersion, and capacity to enhance the immune response [23]. As a result, it is important to mention that both chitin-nanomaterial and nanocomposites are used to manufacture innovative non-woven tissues due to their biocompatibility, biodegradability, non-toxicity, antimicrobial, anti-inflammatory, and hydrating effectiveness, and skin repairing activities [7, 24, 25]. As a matter of fact, these innovating biological dressings can accelerate the healing process at both the molecular and systemic levels. Through their activities, it seems possible not only to facilitate the wound contraction, but also to regulate the secretion of inflammatory mediators, preventing hypertrophic scar formation (Fig. 21.14) [26, 27].

According to our results, chitin nanofibril-lignin non-woven tissue seems to provide a three-dimensional structure that mimics the native extracellular matrix (ECM) as previously reported in Fig. 21.4, while the scaffold is being replaced by the regenerated hard tissues. Moreover, this complex can decrease the bacterial concentration, increasing the production of defensins, thus enhancing the immune response,

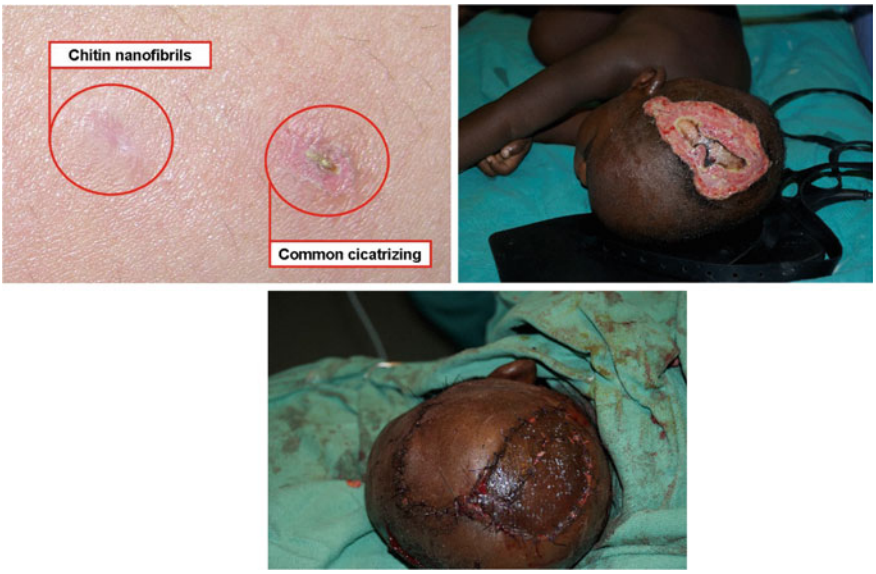

Fig. 21.14 Cicatrized skin treated using a chitin nanofibrils gel without scar formation. (By courtesy of Mezzana [26])

and modulating both growth factors and metalloproteinases activities, while chitin nanofibrils safeguard and regulate the organization of the collagen fibers during the repair of a wounded skin (Fig. 21.15) [7, 25–28]. In summary, the chitin nanofibril-lignin non-woven tissues have demonstrated the capacity to increase the cicatrizing process of people affected by wounds and burns of 1st and 2nd degree without the formation of hypertrophic scars and keloids (Fig. 21.16) [26, 27].

Subsequently, these studies demonstrate that it appears possible to modulate the wound healing process with the aid of specific biomaterials, nanomaterials and

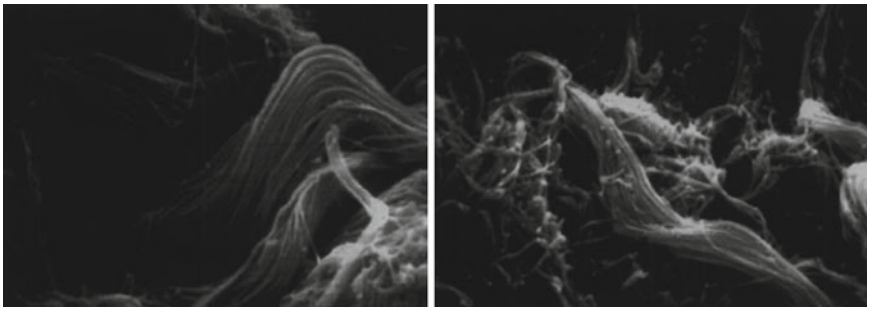

Fig. 21.15 Regular organization of collagen fibers on skin treated with chitin nanofibers: (left) treated; and (right) untreated

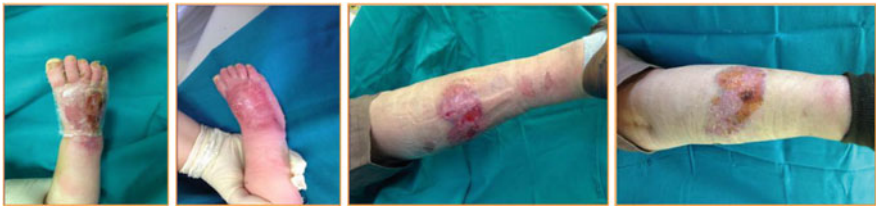

Fig. 21.16 Cicatrizing activity of a non-woven tissue made by chitin nanofibril-lignin complex (By courtesy of Anniboletti et al. [27])

nanoparticles for producing replacement-like process, rather than scar-tissue formation.

Finally, it is worthy to mention that both lignin and chitin, being natural by-product raw materials obtained from agricultural and shellfish waste, such as banana rachis and crustacean by-products, plays a role in preserving natural resources for our next generations and maintaining the equilibrium of the earth ecosystem [6, 7].

21.3 Chitin Nanocomposites

Composite materials are efficient systems made combining two constituents: the reinforcement (filler) and the matrix. They are combined in such a way that the constituents do not dissolve or merge but retain their individual engineering properties [29]. Different types of composites are currently used for a wide variety of applications. They can be classified according to the matrix and reinforcement materials such as polymer-matrix composites, metal-matrix composites, ceramic-matrix composites and carbon/carbon composites. Based on the shape, the filler composites are distinguished in particulate composites, short fiber composites and flake composites. In fiber-reinforced composites, the strong fibers, embedded in the matrix, maintains the geometric arrangement and transmit the load applied to the composite components. As a result of the manufacturing process, the composite materials will have mechanical properties superior to those of the matrix but lower than that of the fibrous reinforcement caused by the interactions between the fibers and matrices.

Biopolymers containing cellulose micro-fibers were the subject of numerous studies during the last decade. Several models were developed to study the interactions between fiber and matrix [30]. Moreover, innovative processing methods were developed to synthesize cellulose-reinforced microcomposites comprising of a high degree of renewable and recycled materials [31]. Interestingly, it was found that natural fibers show a waviness, leading to reduced improvement in properties in comparison to what was predicted using general models applied in composite sector. Hence, a corrective factor was proposed to keep into account this issue [32].

A nanocomposite is a composite in which at least one of the reinforcement dimensions is less than 100 nm (Fig. 21.17) [33]. On the other hand, a nanomaterial is a

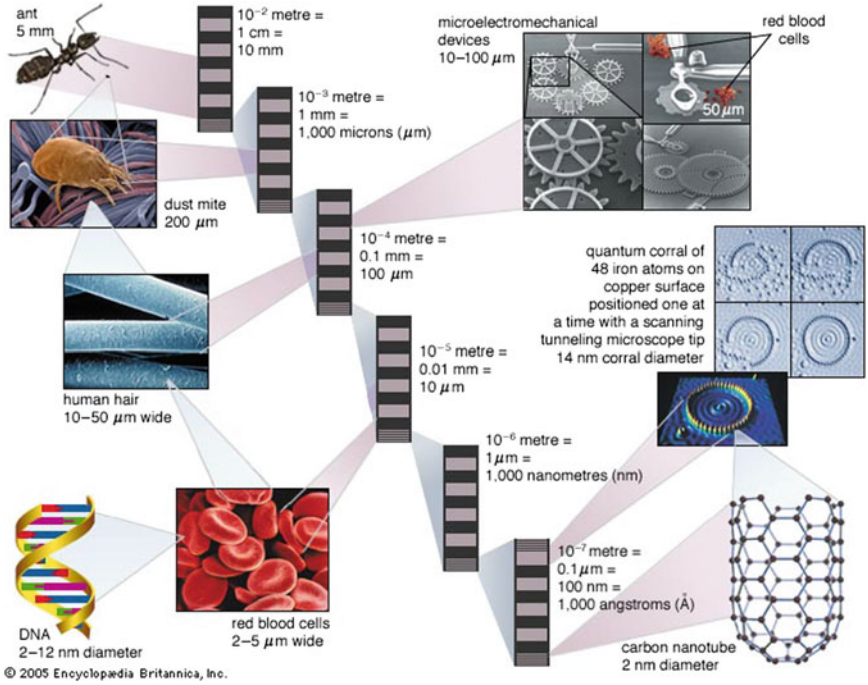

Fig. 21.17 Nanometer dimension [33]

material containing particles, in a bound state or as an aggregate or as agglomerate in which for 50% or more of the particles in the number size distribution, one or more external dimensions is in the range of 1–100 nm [29, 34]. Just to have an idea of this dimension, the smallest bacteria are about 200 nm in length and a DNA double-helix has a diameter of 2 nm. In relations to its properties, the reduced particle size within the nanocomposite promotes reinforcement-matrix interactions, due to the large surface area and short diffusion distance. This plays a significant role in determining the improvements in mechanical properties [34].

One of the main applications of nanotechnology in material science is the development of polymer nanocomposites, reinforced polymers with low quantity nano-sized organic or inorganic ingredients dispersed into a thermoplastic or thermoset polymer. As an example, phyllosilicates-based polymer nanocomposites were manufactured using conventional routes keeping into account the polarity characteristics of polymers and clays [35]. In general, nanocomposites with polymeric matrices are reinforced with small amounts of nanoparticles typically less than 5% by mass, and their manufacturing processes require action at the matrix level to ensure greater dispersion of nanoparticles into the polymer/nanoparticle interfaces [29, 34]. Furthermore, the production of poly(lactic acid)-based nano-composites were investigated in a number of studies in which traditional processing technique [36] and innovative chemical modification routes [37] were compared and/or integrated. Due to the ever

increasing interest on the environmental impacts, the development of nanocomposites reinforced with natural fibers (bio-nanocomposites) is rapidly emerging.

Consumers, in fact, are seeking biodegradable and natural ingredients which, used as drugs or cosmetics, must be effective, safe and capable of making their physical appearance better, giving them a feeling of well-being. Unfortunately, the quality of natural ingredients and fibers depends on a number of variables such as the environment where they are produced, the season, soil quality, and the location from which they originate from within the plant. However, on the one hand, these fibers are hydrophilic, incapable of withstanding high temperatures, low tensile strength, and could easily be contaminated with microorganisms; on the other, they are totally degradable through the environmental enzymes, safe and obtained at low cost.

Moreover, regarding the issues of biodegradability, it is important to remember that once a composite is produced, it almost becomes impossible for the reinforcement to separate from the matrix [29, 34]. Consequently, with respect to the environment and the recyclability of the bio-composite, natural fibers must be used in conjunction with a biodegradable matrix, such as a biopolymer.

For this reason, the use of chitin and its derived compounds as a polymeric material is of great interest due to their availability in significant quantity from the market at low cost, being stable at high temperature with an easy modifiable surface, and totally biodegradable from humans and environmental enzymes [13, 19, 24]. This is also the reason why chitin nanofibrils have attracted particular interest (in addition to their own nanostructure, good thermal stability, non-toxicity and biocompatibility) and making this polymer a promising candidate that can be used in biomedicine for the production of non-woven tissues or biofilms using electrospinning and/or casting technologies. As previously mentioned, it is possible for instance to electrospin a gel made by chitin nanofibrils bound to silver, polypeptides from fish-collagen, and PEO using water as solvent to produce a non-woven tissue capable of regenerating skin tissue in a short amount of time (Fig. 21.18) [19, 27].

On the other hand, it is possible to create a slurry with chitosan and chitin nanofibrils using water and a correct plasticizer to produce a viscoelastic gel that could be used in the manufacturing of films using casting technology [11]. In this case, silver may also bound to chitin nanofibrils, to obtain films which have been shown to possess anti-inflammatory and antimicrobial properties and also beneficial when used in advanced medications (Fig. 21.19) [7, 13, 25, 27].

As previously shown, both chitin nanofibrils-silver-non-woven tissue and the chitin nanofibrils-silver- film have displayed significant antibacterial and cicatrizing activities on burned skin in vivo. Furthermore, it was surprised to discover that films, containing practically no interfibrillary spaces, have demonstrated cell reproduction and survival also, as shown in Fig. 21.20 [11, 27].

During extrusion, the nano-dispersion of chitin nanofibrils in a poly(lactic acid)-based matrix was accomplished with excellent controls in both the final properties and the ability of being processed [38]. This preliminary study has demonstrated that it was possible to incorporate chitin nanofibrils in a bioplastic matrix as an attempt to exploit its functionalities in other applications such as biodegradable packaging films or containers [39]. At the moment, this is an attractive topic for any future research [25].

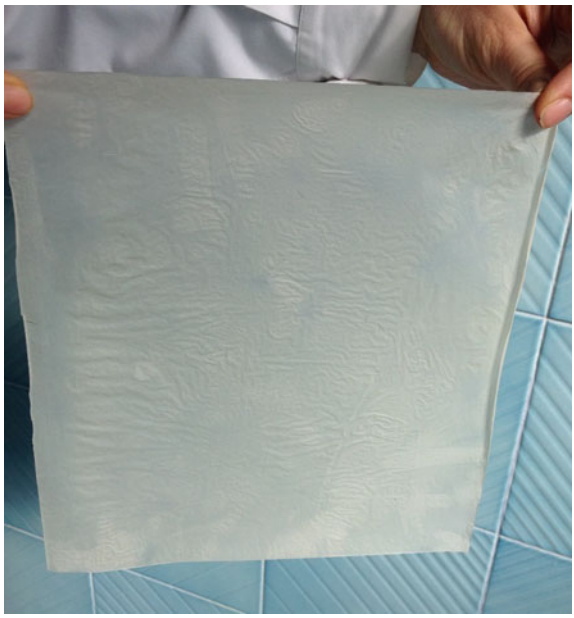

Fig. 21.18 Non-woven tissue made by nanostructured chitin nanofibrils-silver

Fig. 21.19 Transparent film made by nanostructured chitin nanofibrils-silver

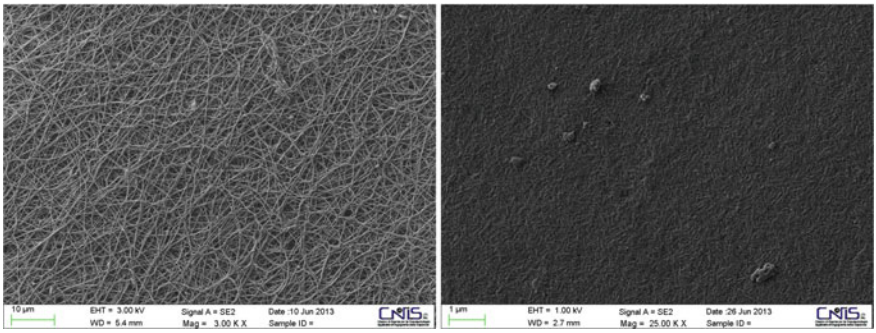

Fig. 21.20 SEM images of non-woven tissue (left) and film (right)

21.3.1 Chitin Nanocomposite and Skin Repair

The skin, as a major organ covering the human body, is a multi-layered biomembrane with essential functions and particular absorption characteristics. Through its outmost stratum corneum layer, it provides a highly efficient barrier for water, and plays an important protective function against microbial and chemical assaults. Common injuries such as wounds and burns can damage this biomembrane and the replacement of this biomembrane is necessary so that its function can be restored in the shortest time possible. Thus, skin substitutes have crucial roles, specifically in the management of deep dermal and full thickness wounds [40].

In fact, more than 6 million patients every year suffers from severe burns and over 0.3 million people die from these injuries worldwide [40]. Additionally, it is reported that chronic wounds affect 6.5 million patients per year in the USA and more than US$ 25 billion was spent by the healthcare system, and similarly around US$ 5 billion in UK [41]. On the other hand, osteoporosis worldwide is projected to increase by 310% in man and 240% in women by the year 2050, affecting 75 million people in EU, USA and Japan, causing more than 8.9 million fractures annually with consequent disability [42]. However, it has been estimated that surgical wounds worldwide affect for around 100 million people per year, while traumatic wounds will affect more than 50 million patients. These figures represent that the wound care industry could potentially have a turnover of around US$ 10 billion, including xenogenic tissue scaffold, dermal substitutes, etc. [43]. Thus, chronic wounds, such as venous and diabetic ulcers, for example, as well as osteoporotic fractures are growing in occurrence with our increasing aging population, and for the increasing awareness and improved diagnosis.

Moreover, a significant feature of all the wounds—approximately 7–10%—is the likelihood of pathological infection that is the result of the procedure or treatment used [41]. Consequently, chronic wounds imposed significant burden on the individual, the healthcare system and the society as a whole. Thus, gaining an understanding into the prevalence and incidence of this category of wounds in relation to population

characteristics and distribution represents an important asset for healthcare planning and resource allocation. In any case, advanced medications and tools, to achieve the best medical treatments are required and necessary as an attempt to solve this important social problem [41]. For these reasons, modern and interactive dressing products are continuously designed and programmed to actively interact with the wound bed, providing optimum environment at the wound dressing interface, maintaining hydration, and preventing infection [44, 45].

The presence of a wet environment is needed in wound healing because the lack of water may affect cell metabolism leading to wound drying. Nevertheless, it is important to remember that wound healing is a biological process which involves inflammation, migration, proliferation and cell maturation activated by keratinocytes and fibroblasts, which cover the wound surface as a new layer, essential during the remodeling process of skin tissues [44]. Thus, collagen for example must be produced in the right quantity with the fibers regularly deposed to avoid the formation of hypertrophic scars and keloids, as it has been previously shown on burned skin treatment [27].

Similar results have been obtained using innovative chitin nanofibrils non-woven tissues, enriched with fish-collagen peptides and nanostructured silver [19, 26, 27]. These specialized tissues, adsorbing exudates, controlling both microbiome growth and the inflammation processes and allowing the diffusion of atmospheric oxygen, have probably increased the tissue regeneration obtained by chitin nanofibrils-non-woven tissues. Thus, the rate of skin healing and the aesthetic repair of wound have been optimized to attain a fast skin repair without infection [7, 25, 27]. Collagen if it is used in the manufacturing of specific nanofiber matrices [45], as reported by other studies, plays a key role in correcting the skin cicatrization processes, while silver could modulate the microorganism's growth [14].

In conclusion, the bacterial-chitin nanofibrils-network of pure chitin nanofibers with high crystallinity, high mechanical strength, wet capability, no toxicity and good skin and environment compatibility, seems to be an ideal candidate for wound dressing if used as non-woven tissue in the manufacture of nanocomposite based on collagen and peptides, as well as the incorporation of lignin and silver nanoparticles [13, 27]. Chitin nanofibrils-peptide composites appear to have reparative properties such as promoting the healing of wound skin tissue, reducing scar formation, and displaying a better water uptake capability than pure chitin nanofibrils, subsequently they facilitate the breathing requirement of injured skin with the growth of primary human fibroblast cells (data not reported). These bio-composite non-woven tissues have shown to modulate the secretion of defensins and MMPs during in vitro experiments [7, 13, 19, 24], while during in vivo studies, they displayed the shortest wound healing time when used to repair burned skin [26, 27].

21.4 Final Remarks

It is necessary to break down the data the composite industry reports in their different segments to gain a better understanding. According to the market research firm Lucintel [46], since 1960 the US composites industry has expanded 25-fold, while the steel industry has only experienced a 1.5-fold increase, and a 3-fold increase for the aluminum industries. On the other hand, the use of polymer nanocomposites for medical applications has gained significant global market with a predicted Compound Annual Growth Rate (CARG) of more than 24% until 2019. This is due to the introduction of innovative bio-based and eco-compatible polymers, contributing to the environment sustainability [47].

Additionally, the market is witnessing a growing demand for biodegradable and environment-friendly nanocomposites, because of the pollution created from the traditional plastic compounds. This is the reason behind the significant growth of nanotechnology and its utilization in regenerative medicine, and the use of nanocomposites produced from natural polymers that is able to mimic biological phenomena at its scale dimension. This could be the reason behind the rapid expansion of novel materials such as chitin and other nanostructured polysaccharides. As a result of their dimension, they can be engineered to imitate skin ECM, repair the neural tissue, mimic the crystal structure of human bone, or to be used as restorative resin for dental applications. Furthermore, the decoding of cellular and molecular mechanisms would enable us to reprogram or instruct cells, creating appropriate and effective nanomaterials and nanocomposites capable of preventing and treating different diseases.

These tissue-systems, therefore, can be engineered to alter their properties-functions in response to specific internal or external stimuli. The goal is to create and produce nanomaterials and nanocomposites characterized by high effectiveness and low toxicity, free of side effects and eco-compatible.

As a result, these innovative biomedical scaffolds must be designed by scientists and technicians who have a deeper understanding into the natural physicochemical activities of biomaterials with a fully integrated knowledge of how the building blocks of human cells, in both healthy and diseases states, function at the molecular level [48]. Thus, it will be possible to establish a bridge between the properties/functions of these scaffolds and the nutritional environmental necessities of the pathological/inflamed tissues, necessary to obtain successful products. This is the goal of our group and the EU PolyBioSkin research project dedicated to the study and identification of innovative nanomaterials, nanocomposites and non-woven tissues for regenerative medicine, made by natural waste ingredients, such as chitin nanofibrils and biolignin. Using these non-woven tissues as innovative carriers, entrapping different and selected active ingredients, it will be possible not only to make medical products to repair diseased skins, but also to produce innovative and effective cosmetics able to regenerate both aged and photo-aged skin.

In conclusion, driving the integration between material chemistry and cell biology, it will be possible to satisfy the continuous request of products useful to maintain the

human wellbeing, contemporary saving the environment biodiversity of our Planet and the natural raw materials for the incoming generations.

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