Sougata Jana
Subrata Jana *Editors*

Marine Biomaterials

Therapeutic Potential

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Preface

This book focuses on marine biomaterials-based biomedical carriers for delivery of therapeutics. Marine biomaterials-based carriers showed wide applications in pharmaceutical as well as biomedical fields for delivery of small and large molecules. Biomaterials-based composites/scaffolds/matrix systems are promising for controlled/prolonged release of drug in target site and prevent/control the premature release of drug/bioactive compounds. The drug substance having short biological half-life often requires frequent dosing, which ultimately may lead to toxicity due to accumulation of excess drug dose. The marine biomaterials-based systems are widely used for tissue engineering and biomedical imaging. So, the marine-based biomedical carriers are promising in the area of drug targeting and biomedical engineering to the target site.

This volume (II) is specially focused on various marine biomaterials and their therapeutic potential. The different chapters of this book is highlighting the therapeutic potential of marine collagen, management of bone disorders, gene delivery, marine natural compounds in immunomodulation, theranostic applications, tissue engineering, and regeneration.

Chapter 1 contains basic information on marine biomaterials and its resources, categories, and applications.

Chapter 2 deals on the possibilities of crosslinking the polysaccharides from marine resources along with its organization and different types of crosslinking methods. Atanase et al. discussed about some important polysaccharides such as chitosan, chitin, k-carrageenan, hyaluronic acid, alginic acid/alginates, agar/agarose, and fucoidan.

Chitosan is a biocompatible and biodegradable marine biomaterials, and it has potential therapeutic application. Chapter 3 focuses on chitosan and chitooligosaccharides along with their preparation, characteristics, and potential application as therapeutic agents.

Among the different marine biomaterials, collagen is highly used as marine biomaterials for various applications, due to their biodegradability, biocompatibility, low antigenicity, confirmed structure, and biological properties. Chapter 4 in this book is dealt with marine collagen and the applicability for the delivery of therapeutics.

Gene therapy is a branch in medical science with therapeutic potential for treating a disorder at its genetic root. The gene therapy is generally depending on the carriers

or vehicle's ability to selectively and efficiently deliver gene to target site with minimal or no side effects. Chapter 5 focuses on delivery of gene using marine biopolymers as efficient carrier.

The polymeric hydrogels have received considerable attention as leading candidates for engineered tissue scaffolds due to their unique compositional and structural similarities to the natural extracellular matrix. Chapter 6 is highlighted on different hydrogel scaffolds based on alginate, gelatin, and 2-hydroxyethyl methacrylate for tissue regeneration.

Now-a-days, tissue engineering is considered one of the most important therapeutic strategies for regenerative medicine. It is an important alternative approach by using of scaffold materials with combined engineering and biochemical/physicochemical methods for either replacing or improving of lost tissues or those are become nonviable due to disease or trauma. The applications of tissue engineering based on marine biomaterials are covered in Chap. 7.

Currently, theranostic approach is important for various functions ranging from diagnosis to treatment with accurate targeting of cancer-specific cells. The theranostic systems are attractive and useful for drug targeting and biomedical imaging are being carried out in a single platform. Chapter 8 emphasizes on the theranostic applications of marine biopolymers.

In Chap. 9, authors have highlighted on the marine biomaterials as carrier of drugs/biomolecules for managing the bone disorders. Different marine biomaterials are used as drug delivery vehicles for the treatment of various bone disorders. This chapter provides an overview of presently used marine materials as bone graft substitute vis-à-vis as carriers in local drug delivery systems.

Chapter 10 provides a detailed review on some important marine natural products discovered so far along with their immunomodulation activities against various infectious, chronic, and non-chronic diseases. The authors also highlighted the information about challenges and future perspectives of those natural products.

Recent development and biological properties of different peptides derived from a variety of living organisms, including microbes, plants, and marine animals are focused in Chap. 11. Besides these, authors also highlighted on in vivo, in vitro, and clinical trials along with their potential applications for the treatment of diseases.

This book is a collection of most updated recent progress in the field of marine biomaterials for therapeutic applications, which is written by the experts from their own field and will be very useful for the students, researcher scholars, and scientists in the area of pharmaceuticals, biomedical engineering, and materials science.

We express our sincere thanks to all authors for their contribution to this edited book. We also thank the publishers for their continuous support in completion of this reference book.

Kolkata, West Bengal, India — Sougata Jana
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About the Editors

Sougata Jana has completed his Ph.D. in pharmaceutical technology from Maulana Abul Kalam Azad University of Technology (MAKAUT), West Bengal (Formerly Known as WBUT), India. He is engaged for fourteen years in the field of pharmacy, including teaching, research, health services, etc. He has published 30 research and review articles in different national and international peer-reviewed journals. He has also edited 10 books and published more than 45 book chapters in different edited books from internationally renowned publishers. He is the reviewer for various peer-reviewed international journals. He is a life member of the Association of Pharmaceutical Teachers of India (APTI) and has an associateship with the Institution of Chemists (AIC), India. He successfully guided 17 postgraduate students for their research projects. He is working in the field of drug delivery science and technology, including modification of synthetic and natural biopolymers, microparticles, nanoparticles, semisolids, and interpenetrating network (IPN) system for controlled drug delivery.

Subrata Jana is presently working as an associate professor at the Department of Chemistry, Indira Gandhi National Tribal University (Central University), Amarkantak, Madhya Pradesh, India, and his current research focuses on design and synthesis of artificial receptors for the recognition of anions, cations, and biomolecules along with biodegradable polymeric-based carrier systems for the delivery of drug molecules. So far, he has published ~40 research papers in peer-reviewed international journals and contributed more than 20 book chapters in different edited books

published by internationally renowned publishers. He also served as reviewer for various international journals.

He has obtained his PhD in organic chemistry from Indian Institute of Engineering Science and Technology (IIEST), Shibpur, India. Then he worked with Professor (Dr.) Fraser Hof at the University of Victoria, Canada, and Dr. Kenneth J Woycechowsky at the University of Utah, USA, as a postdoc. Overall, he extensively studied on supramolecular behavior of the host–guest interaction and synthesis of different heterocyclic moieties.

Marine Biomaterials: Resources, Categories, and Applications

1

Hassan A. H. Ibrahim, Waleed M. M. El-Sayed, and Mostafa M. El-Sheekh

Abstract

The marine ecosystem is a unique and complicated system that is characterized primarily by the great biodiversity, representing the richness of diverse habitats and the variations of life forms. It involves various marine organisms with great chemical diversity, and they are rich sources of secondary metabolites relative to terrestrial organisms. The oceans thus always offer excellent opportunities for discovering valuable materials from various organisms. Such compounds contain a wide range of chemical structures and functions, which for more specific potentialities are wider. The produced compounds have a vital role in the human and animal life and are widely used as antimicrobial, antioxidants, antiviral, anticancer, and food and feed and in food and pharmaceuticals industry. However, in many fields such as food, cosmetics, dietary supplements, animal feed, bioactive packaging, and industrial products, as well as in high-tech biomedical sectors, applications of novel marine molecules are now found.

Keywords

Biodiversity · Marine ecosystem · Marine biomaterials · Biopolymers · Bioceramics · Sulfated polysaccharides

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1.1 Introduction

Oceans cover the highest percentage of the surface of the world and abound in the biodiversity of both marine and coastal habitats and contain 97% of the Earth's water. Therefore, the oceans are critical to the life on Earth. They have a principal role in the global conservation of nutrients and the climate control and have a wide variety of benefits for people. Ultimately, life in oceans provides about 33% of the oxygen we breathe, and human fish consumption accounts for 16% of global supply of animal protein. Moreover, it is especially significant as a source of protein for populations in developing countries (IUCN, Europe).

On the other hand, marine organisms and microorganisms (such as bacteria, fungi, sponges, microalgae, seaweeds, crustaceans, mollusks, vertebrates, and fish) are made up of fundamental substances and secondary metabolites compared to terrestrial organisms with a vast array of unique properties. However, this reflects in the evolutionary process of natural self-selection and progression (Pandey 2019). Thus, oceans have a considerable interesting forum for developing novel biomaterials, with both economic and environmental benefits and can explain their possible applications in the biomedical industry, drug delivery, health care, food processing, and tissue engineering devices (Bhatnagar and Kim 2010).

Generally, marine raw materials are categorized as polysaccharides (such as agar, alginates, carrageenans, chitin, chitosan, etc.), proteins, glycosaminoglycans (e.g., collagen, chondroitin, heparin, and hyaluronic acid), and ceramics (such as calcium, phosphorus compounds, and biosilica) (Silva et al. 2012).

Many biomedical and industrial applications by biomaterials developed by marine organisms have been increasingly investigated (Rinaudo 2006). In addition, the study of several biomolecules has led to comprehensive preclinical investigations that justify clinical trials in many therapeutic fields, involving viral infection and tumor formation (Dembitsky et al. 2005).

1.2 Unique Characteristics of the Marine Ecosystem

Seawater occupies about 70% of the Earth, and despite occupying less than 10% of the Earth's surface, coastal waters and oceans provide around 30% of marine productivity and represent 90% of the marine fisheries. For 80% of organic matter, they are the burial site, and 90% of sedimentary mineralization and nutrient cycling takes place there. They also supply the sink with up to 90% of the suspended load in the world's rivers, along with many associated contaminants. On the other hand, the seas and oceans have an average depth in excess of 3500 m, and more than 60% of the Earth's surface lies below more above 200 m of the seawater (Millennium Ecosystem Assessment 2005).

The open ocean species, like microorganisms, crustaceans, billfish, seabirds, tunas, sharks, etc., are widely distributed and exist in the most areas of the ocean. So, the oceans are thought to be much more dynamic and complex than previously assumed. Scientists are only beginning to understand the organisms' life history and

various metabolic forms and how these habitats operate and interact with the above water column (UNEP 2006). The vast majority of marine biodiversity records come from shallow waters (< 200 m) or the seabed, and the pelagic ocean, which is the greatest source on Earth in which life can exist (> 109 km^3), is recognized as particularly underrepresented in global databases (Webb et al. 2010).

Where primary terrestrial producers tend to be large and sessile, primary marine producers tend to be small and mobile and thus subject to fluid transport and spatial mixing processes. Photosynthesis is limited to the upper sunlit parts of the ocean, which are much less productive over vast areas than the soil, since growing phytoplankton strips nutrients from the surface waters (Bar-On et al. 2018).

Development and use in the sea can be decoupled with fixed carbon being consumed several times before being completely remineralized, and while the available organic matter is increasingly scarce with depth, it supports numerous pelagic and benthic ecosystems. Consumers, grazers, and carnivores, who can be many times larger than their terrestrial counterparts, have a much greater variety of life-history features on the other end of the aquatic food web than terrestrial ones. Most marine predators have extremely high reproductive efficiency, producing millions of offspring, particularly among older and larger members of their populations. This may help them to survive overexploitation but also makes populations highly variable, difficult to predict, and vulnerable to threshold effects (Kallmeyer et al. 2012).

Predation encouraged the evolutionary advantage of defensive and reinforcing structures such as biomineralized skeletons with bristles and spines, fostering coevolution among predators and prey and, coincidentally, the possibility of fossilizing species. The practices of animal feeding and bioengineering encouraged and altered the biogeochemical cycles. For example, animals that feed on plankton converted slow-sinking cells into rapidly sinking fecal pellets, providing a source of organic-rich sediments and altering their biogeochemistry (Meysman et al. 2006).

It is precisely phytoplanktonic species that increased in size and formed biomineralized structures, allowing them to sink faster and thus shift the output of exports from the surface ocean. Concentrating biomass into fewer yet larger species increased the free oxygen supply. The evolution of filter feeding provided a mechanism for clearing microbial populations and providing a relatively non-turbid photic zone where primary production of the dominated algae could occur (Basu and Mackey 2018).

1.3 Marine Biodiversity and Ecosystem Functioning

Marine biodiversity is extremely ancient, and life is likely to have arisen in a marine environment between 4500 and 3500 Mya (million years ago), with 3000 Mya chemical autotrophic prokaryotes and photosynthetic cyanobacteria and around 2000 Mya eukaryotic cells (Cavalier-Smith 2006). Observably, cyanobacteria dominated life in the water column, the small size of which limited their sinking rate and maintained positive feedback maintaining cyanobacterial dominance,

turbidity, and anoxia (Butterfield 2010). However, life on the seafloor was characterized by microbial mats (Bottjer 2005). The capability of ecosystems to supply the human with the services they require to exist is frequently stated to be based on biodiversity. The significance of biodiversity in ecosystem functioning, as well as the relative responsibilities of various functional groupings, has proven to be incredibly difficult to comprehend (Hooper et al. 2005).

In the coastal and shelf seas, marine biodiversity is the highest supported by the presence of diverse sedimentary habitats, rocky and biogenic reefs, kelp forests, and seagrass beds. Some of these are highly specialized ecosystems. Specialized collection of species, for example, inhabits the intertidal zone ranging from rocky to sandy shores, and wave exposure is a key determinant of the population living on a specific shore (Lotze and Worm 2009).

The world's oceans provide human with critical ecosystem services. Worm et al. (2006) conducted a meta-analysis of published studies to see if the level of marine diversity had an impact on the services of certain ecosystem, like nutrient cycling, productivity, resource usage, and ecosystem stability, and thus whether marine degradation was affecting ecosystems' ability to provide services. Increased genetic or species variety was found to be beneficial in the study.

Since 2002, 18 global-scale studies of marine biodiversity hotspots have been published. Species richness peaked in Australia, Southeast Asia, Japan, South Asia, Southeast Africa, the Caribbean, and the United States' southeast region. Endemism hotspots have been discovered in South Africa, Australia's east and west coasts, and remote locations such as Hawaii, Easter Island, New Zealand, and Antarctica. In the western Caribbean, South Madagascar, Chagos Islands, Maldives, Lakshadweep, Sri Lanka, South Japan, the East China Sea including Taiwan, Coral Triangle, New Caledonia, Vanuatu, and North East and North West Australia, there exist hotspots of both high species richness and endemism (Jefferson and Costello 2020).

In particular, the most critical areas to biodiversity are the deep sea, open ocean hotspots, seamounts, hydrothermal vents, cold water corals, sponge fields, and others (UNEP 2006). Marine protected zones are critical for preserving ocean biodiversity and maintaining productivity, particularly for fish supplies (Owen 2005). However, the following text will refer to the biodiversity in these areas:

1. *Deep sea* – in which life is diverse and specialized with organisms living in constant darkness at high pressures and low temperatures. However, the number and variety of organisms per unit area or volume are generally low compared to shallow coastal systems, with diversity tending to decline with increasing depth. The proportion of deep-sea fauna is lower than for other marine systems and unlikely to exceed the number in shelf seas. Life in the deep sea is comparably young, as for most of Earth's history, the deep sea has been inhospitable to life (Widdicombe and Somerfield 2012).
2. Mid-water column – where beneath 200 m remains a tremendous enigma that scientists have just recently begun to investigate. Organic waste that floats down from surface waters like falling snow feeds the food chain. Many tiny animals with unique adaptations to use such trash, besides strange-looking fish that

dangle light lures to catch naive prey and the elusive octopus, live in the mid-water column (UNEP 2006).

3. *Deep seabed* – where the place was barren and remembered as the very cradle of life, with a great variety of thrilling geological and biological extremes. Challenger Deep in Marianas Trench, at 11,034 m, is the deepest location on Earth. This diversity of characteristics and habitats leads to that 98% of all marine species are thought to support the seabed and that more species live in deep seabed environments than in any other marine environments combined. The wide range of seabed ecosystems produces diverse organisms and life forms with remarkable adaptations to such harsh conditions, ranging from generalists digesting sediments to highly specialized species living primarily on whale bones and feeding on them. In this area, active communities of bacteria can be found hundreds of meters beneath the seabed, feasting on old sediments (Owen 2005).
4. *Volcanic vents* – in which the origin of life is believed to have originated, far from the rays of the sun, receiving its energy from the superheated minerals that escape from the molten center of the Earth. It is estimated that up to ten million creatures will survive within the sediments of the continental slope and abyssal plains. Mysteries of the farthest and deepest oceans revealed submarine canyons are common along the slopes of most continental borders and certain oceanic islands, yet each one is distinct due to its structural intricacy (Einarsson 2008).
5. *Canyons* – where the high habitats diversity within canyons results in a very high density and species diversity compared to the continental slopes. Species include many filter feeders like sponges, hydroids, corals, sea pens, sea cucumbers, brittle stars, tube worms, and anemones. Many significant species upon commercial level such as crab, shrimp, lobster, flounder, and tilefish are often found in canyons, because they often serve as important nurseries for them. Additionally, the high productivity of canyon makes them valuable feeding grounds for many pelagic animals (UNEP 2006).
6. *Seamounts* – under sea mountains of volcanic origin, vary often in the size and height. Some of them rise from the seafloor about 100 m, while others tower 3000 m or more. Amazingly, the world's oceans include more than 100,000 seamounts, but less than 200 have been studied in depth so far. Seamounts communicate with the surrounding seas, trapping plankton, and increasing the productivity, showing them as valuable hotspots for many open ocean and deep-sea species to feed, breed, and spawn (Owen 2005).
7. *Hydrothermal vents* – where temperature may reach to up to 400 °C and sulfur-rich emissions are converted into energy by bacteria and primitive microorganisms, which feed a unique ecosystem of worms, crabs, mollusks, shrimps, anemones, soft corals, and other species. Many species are unknown to scientists, and more than 75% occur at only one site. Nevertheless, giant tube worms have adapted to live near these vents, without mouths or digestive systems, growing nearly 2.5 cm in 10 days (UNEP 2006). *Prochlorococcus* has been identified in the photic zone as a tiny photosynthetic cyanobacterium,

which is likely the most abundant organism on Earth accounting for 20% of the Earth's atmosphere oxygen (Colín-García 2016).

8. *Cold filling* – where cold and chemical-rich water (or methane) flows from the seabed, or where undersea landslides or streams uncover chemical/gas-rich sediments. These chemicals and gases help special species and chemosynthetic habitats. There is some overlap but not much between the vent and cold seep cultures. Of the 211 cold vegetable species reported so far, 13 species occur in both vegetation and vents. Scientists have just realized that the populations of cold seep and gas hydrate ultimately rely upon sunlight (Owen 2005).
9. *Cold water corals* – from the poles to the deep waters of tropical islands (on seamounts and in fjords, submarine canyons, coastal cliffs, banks) and mid-ocean ridges; they have been widely distributed in all oceans. They live in seawater temperatures ranging from 4 to 13 °C and depths of 39–5630 m deep. Despite sunlight, these corals can survive by catching tiny particles of food suspended in the water column. Nonetheless, some corals, such as *Lophelia pertusa*, may develop huge and intricate reefs and resemble to their tropical shallow-water cousins; other corals can form lush thickets or forests on the seabed or seamounts, usually in locations with strong currents. However, these coral habitats, on the other hand, may support a wide range of related species as well as juveniles of many commercially important fish species (UNEP 2006).
10. *Sponge reefs* – can grow to immense size and age; they could be over 100 years old and weigh up to 80 kilograms. Many species, including commercially important species like redfish and cod, use sponge aggregations to create a three-dimensional structure on the seabed that provides protection, hunting grounds, and refuge. Near sponge fields in the North Atlantic as they did on the surrounding seabed, scientists have discovered roughly twice as many species. Moreover, deep-sea sponges are slow-growing, fragile, and highly vulnerable to damage by bottom fishing gear (Owen 2005).
11. *Coastal systems* – such as salt marshes, mangroves, and seagrass meadows which are capable of absorbing or sequestering carbon at up to 50 times that of the same tropical forest area. These systems may contain up to five times the total carbon deposits stored in the tropical forests (Jefferson and Costello 2020).

Finally, biodiversity loss is being driven by a multitude of anthropogenic stresses on multiple spatial and temporal scales. These stresses act simultaneously to reduce taxonomic and functional diversity (Widdicombe and Somerfield 2012). However, the major threats to marine biodiversity are numerous: habitat degradation, climate change, and ozone depletion; effects of fishing, nutrient runoff, marine pollution, and species invasions; watershed alteration; and the physical alteration of coasts; etc. All these threats need global legalizing volition to conserve marine biodiversity (Owen 2005).

1.4 Categories of Marine Biomaterials

Several marine origin materials, including biopolymers (such as polysaccharides and proteins), bioceramics, unsaturated fatty acids, natural pigments, toxins, and microbial bioactive substances (antibiotics, enzymes, nanoparticles, etc.), have been extracted and examined in the tissue engineering, food processing, drug delivery, and other vital applications based on their main superior properties (Shahidi and Alasalvar 2011). However, these polymers are playing predominant biological role in the producer habitat (Fig. 1.1).

1.4.1 Biopolymers

The organisms of the sea biosynthesize a large number of biopolymers, which are classified mainly into three classes: polysaccharides, proteins, and nucleic acids (Navarro et al. 2019). In general, polysaccharides are termed as biopolymers constituted from carbohydrate monomers (hexoses) connected together by glycosidic bonds. In particular, chitin, agar, alginate, and carrageenans are the most representative polysaccharides in the marine environment (Navarro et al. 2019). Other polysaccharides also have been obtained from marine animal or bacterial sources. For instance, ulvan and fucoidan are two sulfated polysaccharides that can be found in brown and green algae, respectively (Li et al. 2008). However, Fig. 1.2 represents a side of common marine polysaccharides.

1.4.1.1 Collagen

Collagen is the most abundant protein in the vertebrates, and it is essential to several organs such as bones, muscle, skin, and cartilage. Fundamentally, it acts as a structural matrix. Fish-derived collagen is also a major source of imitation of the

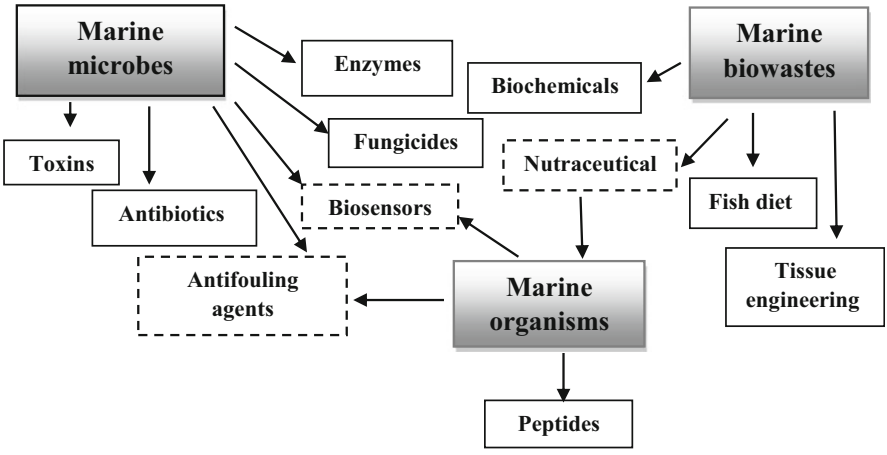
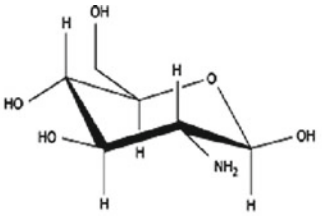
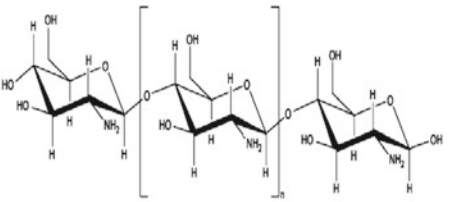


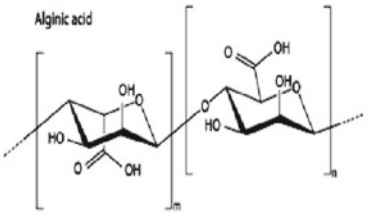
Fig. 1.1 A side of bioactive substances diversity from marine environment



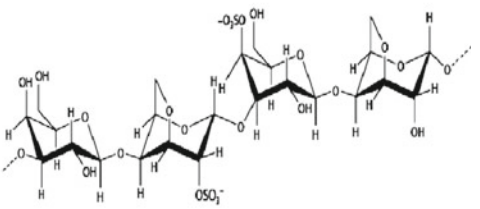
Glucosamine



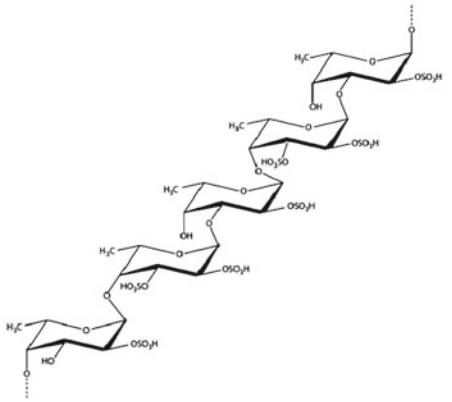
Chitosan



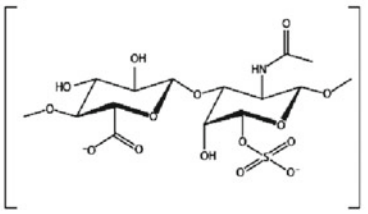
Alginic acid



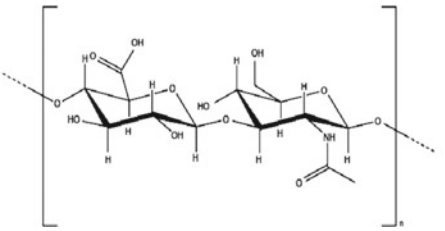
Carrageenan



Fucoidan



Chondroitin sulphate



Hyaluronan

Fig. 1.2 A side of famous structures of common polysaccharides

same quality obtained from other sources. Also, it is used in many applications as pharmaceuticals, nutraceuticals, cosmeceuticals, and foods. In addition, it is widely used in the biomedical applications, particularly in the bone diseases (Kim and Mendis 2006).

Typically, collagen has been obtained from a bovine source which carries a high risk of encephalopathy or spongiform transmissible encephalopathy. So, the marine source is very significant. It has been isolated from fish waste materials like bone, skin, and fins (Nagai and Suzuki 2000); muscles and skins of marine animals (Sikorski and Borderias 1994); the marine sponge (*Chondrosia reniformis*) (Swatschek et al. 2002a); jellyfish (Ehrlich et al. 2010); cuttlefish (Nagai et al. 2001); squid skins; and marine sponge (Swatschek et al. 2002b).

Structurally, collagen is formed from three chains of extended protein, which wrap themselves around each other as a triple helix. Heat denaturation makes collagen easy to convert into gelatin. Both collagen and gelatin are specific proteins, and their content of nonpolar amino acids such as glycine (30%), alanine (10%), and proline (10%), as well as the significant presence of hydroxyproline makes them unique with specific properties (Mendis et al. 2005).

A recombinant collagen is an effective form of processing collagen on a wide scale (Cregg et al. 2000). In spite of the expression system of *Escherichia coli* incorporating many benefits, its shortcomings in expressing eukaryotic proteins make it restricted to develop the small fragments and lack post-translation modifications to the proteins produced. So, various eukaryotic systems have been modified to accomplish this mission, such as *Pichia pastoris* yeast and insect cells of mammalian and tobacco (Cregg et al. 2000). Also, *P. pastoris* is an important development method for very large and complex proteins, such as collagens (Macauley-Patrick et al. 2005).

1.4.1.2 Chitin and Chitosan

After cellulose, chitin is the second most abundant natural polymer. It is a natural polysaccharide found in exoskeletons of arthropods (shrimp, crab, lobster, jellyfish, coral, butterfly, fungi, etc.), and endoskeleton of mollusks. For the chitosan production, the shells of the marine crustacean are widely used as basic resources (Shahidi and Abuzaytoun 2005). Commercially, the chemical hydrolysis and enzymatic methods are generally applied to isolate chitosan from shells of marine crustaceans, which are quite inexpensive, and chitin and chitosan are also used to produce glucosamine (Peniche et al. 2008). Structurally, chitin consisted of a linear chain from units of N-acetyl-D-glucosamine (Kumar et al. 2006), while chitosan is formed from D-glucosamine (70–90%) and units of N-acetyl-D-glucosamine (10–30%), connected by b (1R4) glycosidic linkage (Kumar 2000).

The degree of deacetylation determines the structural difference between chitin and chitosan, i.e., the polymer chain deacetylated unit ratio, since the structural structure is identical for both polymers. The deacetylation degree value for chitosan varies from 50 to 90% making it soluble in dilute acetic acid solutions, in which amine groups in glucosamine units are converted into the soluble protonated form (Pillai et al. 2009). Amazingly, chemical alterations on chitin/chitosan have proven

effective for constructing a large range of useful derivatives with potent mechanical, solubility, and biological features due to the presence of amino groups (Mourya and Inamdar 2008).

Chitosan is a commercially available product made from chitin separated from crab exoskeletons. Basically, the chitin is isolated from its resources via three important steps: demineralization to remove calcium carbonate by hydrochloric acid, deproteinization (using 2% NaOH solutions), and depigmentation (removal of pigments by acetone or other solvents) (Hayes et al. 2008).

1.4.1.3 Agar

Agar is a complex polysaccharide found in red algae's cell wall, namely, agarophytes, including *Gelidium* and *Gracilaria* species. Commercially, agar is derived primarily from the *Gelidium*, *Gracilaria*, *Acantkopeltis*, *Pterocladia*, and *Ceramium* species (Li et al. 2008). Structurally, agar is a polysaccharide consisting of neutral agarose and charged agaropectin. Moreover, it is a complex mixture of water-soluble galactan derivatives. Specific substituents, such as methoxyl, sulfate, and pyruvate groups, increase their strength. The existence of ester sulfate and ketal pyruvate in the backbone gives the polysaccharide an ionic character (Pereira-Pacheco et al. 2007).

The agar production starts with pre-treating the algae for bleaching off coloring matter and eliminating the lipidic matter, followed by extraction of agar, purification, dehydration, and then desiccation, which will impair final polysaccharide quality (Rinaudo 2008). Generally, agar is obtained when the alga is cooked in water, and the remarkable yield loss and decrease in the resulting agar's rheological properties are typically related to elevated extraction temperatures and prolonged extraction time (Kumar and Fotedar 2009). Yield can be improved by adding a small amount of phosphate, which is normally pyrophosphate (Rinaudo 2008). This gel is filtered by freezing and thawing to remove water, which contains salts, pigments, and polysaccharides. The final extract quality is defined by its rheological properties, i.e., a gel will be formed from a high-quality agar at 1.5% solution with strength greater than 700 g/cm^2 (Kumar and Fotedar 2009).

1.4.1.4 Fucoidan

Fucoidan is a sulfated polysaccharide present in a variety of brown seaweed species, for example, *Fucus vesiculosus*, *Macrocystis pyrifera*, *Ecklonia kurome*, *Chorda filum*, *Himanthalia lorea*, *Bifurcaria bifurcate*, *Ascophyllum nodosum*, and *Hizikia fusiformis* (Shiroma et al. 2003), besides sea cucumber species. Structurally, fucoidan is mostly generated from brown algae and contains significant amounts of L-fucose and sulfate ester groups (Li et al. 2008).

During the previous few years, many fucoidans' mechanisms have been detected, and a variety of biological behaviors has been discovered. Fucoidan is a dietary supplement ingredient that has been used for a variety of biological and medicinal purposes (Fitton 2011). The marine fucoidan from *Ascophyllum nodosum* was tested for antitumor, immunological, cosmeceutical (Fitton et al. 2007), and anticoagulant properties (Colliec et al. 1991).

1.4.1.5 Alginate

Alginate is found in the cell walls of the brown seaweeds, as part of a broad family of glycans that make up such community of species. Quantitatively, it represents as the largest polysaccharide in brown algae, adding up to 45% of these seaweeds' dry weight. It is important for its versatility, having both mechanical and structural functions, and ionic roles in brown algae (Nyvall et al. 2003). Mainly, the commercial alginates are isolated from the *Laminaria*, *Ascophyllum*, *Macrocystis*, *Ecklonia*, *Sargassum*, *Lessonia*, and *Durvillaea* species (Gomez et al. 2009).

Brown algae alginates differ chemically from one genus to the next, as well as between distinct algae tissues. Quantity, composition, and sequential structure of the alginates are influenced by various factors including organism taxa, tissue age, season, form, and environmental impacts (Rioux et al. 2007).

Although the algal alginate is well-documented for commercial reasons, certain bacteria can create polysaccharides that are similar to alginate (Rehm and Valla 1997), which are produced mainly by *Azotobacter* and *Pseudomonas*, to protect vegetative cells from desiccation and unfavorable conditions (Sabra et al. 2001).

Practically, the alginate extraction began with an acidification step to change the salts of alginate dissolved in the water into insoluble alginic acid form. In addition, this step removes contaminant glycans, such as fucan and laminaran. Afterward, this is followed by alkaline extraction, using NaOH to convert the insoluble alginic acid into soluble sodium alginate that is extracted by precipitating them from the solution and then dried well (Vauchel et al. 2009).

1.4.1.6 Carrageenan

Carrageenan is a class of linear sulfated polymers isolated from certain red algae species, mainly from the genera *Chondrus*, *Eucheuma*, *Gigartina*, and *Iridaea*. Carrageenan accounts for 60–80% of its dry weight in addition to protein (10–47%); there are florid starch and different metabolites and compounds like vitamins, phenols, and essential oils (Fleurence 1999).

Structurally, carrageenans are linear polymers consisted of a backbone derived from galactose units of alternating link 3, b-D-galactopyranose and 4, a-D-galactopyranose with regular but imprecise structures, depending upon their source and extraction conditions. The number of sulfate groups per basic disaccharide unit after alkaline modification (conversion of precursor molecules into their final commercial product) distinguishes three main types of carrageenan: k (kappa), i (iota), and l (lambda) are disaccharides with one, two, and three sulfate groups, respectively (Falshaw et al. 2001).

Kappaphycus and *Eucheuma* species are the main sources of iota and kappa carrageenan, while many marine algae belonging to the Gigantinaceae family are the main source of the lambda carrageenan. Chemically, kappa-2 carrageenan is a hybrid polysaccharide that contains both kappa and iota units in the same polymer chain (Falshaw et al. 2003).

Both kappa and iota carrageenan gels are hard and brittle, whereas iota carrageenan gels are flexible and soft (Yuguchi et al. 2003). Although all varieties of carrageenan are water-soluble, only the lambda form is soluble at low temperatures

and so rarely forms gels. Solubilization in hot, mild alkaline solutions is used to extract carrageenan from red algae (Hilliou et al. 2006). The process of carrageenan extraction starts up with water and/or mild alkali.

However, several purification strategies are already defined, including dialysis, previous extraction of other components, and reprecipitation among others. Nextly, the produced carrageenan has widely been applied in modern biotechnological methods, profiting from its high potential for chemical alteration, fascinating visco-elastic and biological properties (Silva et al. 2010).

1.4.1.7 Laminarin

Some brown algae species produce laminarin, a linear storage β-1,3-glucan that can be removed. In seasonally variable habitats, its chemical storage form of carbon allows perennial brown algae to decouple growth from photosynthesis. The majority of these plants develop in the winter as seasonal anticipators, reliant on laminarin remobilization, whereas growth has slowed as the storage pool has been filled. Due to this significant ecological importance, a good and precise method for determining and quantifying laminarin is necessary. As a result, a simple and successful cold water extraction process was created in conjunction with a new spectrometric quantitative liquid chromatography-mass spectrometry (LC-MS) (Graiff et al. 2016).

The laminarin content from brown algae on dry basis has been recorded to a level of 35% that differs according to species, harvesting season, environment, and extraction process. Laminarin has been investigated and conducted in various biofunctional activities, including antitumor, anti-inflammatory, antioxidant, and anticoagulant. After appropriate chemical modifications and sulfation methods, laminarin's biofunctional activities can be enhanced (Kadam et al. 2015).

1.4.1.8 Ulvan

Ulvan is a polysaccharide cell wall and from 9 to 36% of its dry weight is primarily made up of sulfated rhamnose, uronic acids (glucuronic acid and iduronic acid), and xylose. The cell walls of *Ulva* species contain three additional polysaccharides (cellulose, xyloglucan, and glucuronan), which account for up to 45% of the dry weight biomass (Lahaye and Robic 2007). Ulvan is the only cell wall polysaccharide that contains both rhamnose and iduronic acid, out of the four polysaccharides found inside *Ulva*. Rhamnose is studied for its impact on dermal biosynthesis pathways and plant immunity (Adrien et al. 2017).

Since its uronic acid content is related to a sulfated neutral sugar, ulvan is considered a candidate for the regulation of processes and functions performed by mammalian polysaccharides. Ulvan could be used in biomaterials (wound dressings, tissue engineering, biofilm prevention, and excipients), nutraceuticals (antivirals, antioxidants, antihyperlipidemia, anticancer, and immunostimulants), foods, and agricultural field (Venkatesan et al. 2015; Cunha and Grenha 2016). The choice of extraction conditions usually depends on the physicochemical characteristics of the ulvan molecule and its particular interactions with other components of the plant cell wall (Robic et al. 2009). Co-extraction of contaminants can result in more intensive

downstream purification procedures, depending on the intended application of the ulvan extract (Kidgell et al. 2019).

1.4.1.9 Glycosaminoglycans

Glycosaminoglycans (GAGs) are natural polysaccharides that are linear, complex, and polydisperse. They are made up of a repeating disaccharide unit made up of a hexose and a hexosamine (Zierer and Mourao 2000). The existence of sulfated GAG in a numerous marine phyla-like sponges (Porifera) and various groups of fish, especially in commercially important species such as sharks, skateboard, codfish, salmon, and trout, is now well established (Im et al. 2009). Due to their unique chemistry, marine-derived GAGs are largely investigated due to their medicinal activities (antimicrobial, anticoagulant, and antitumor) and as novel biomaterials having various applications such as bioadhesive molecules, tissue engineering, and regenerative medicine (Medeiros et al. 2000).

Generally, GAGs are extracted from selected proteoglycan bearing tissues following quite general methods via the usage of appropriate solvent conditions for sufficient isolation from the tissue, with 4 M guanidine HCl concentrations. In addition, the employment of protease inhibitors is highly recommended to hinder early proteoglycan degradation. After removal from the protein core, various separation processes such as sodium dodecyl sulfate polyacrylamide gel electrophoresis, membrane separation, and chromatographic separation may be used (e.g., size exclusion chromatography). Further purification can be accomplished with dialysis, gel filtration, and ultrafiltration (Yanagishita et al. 2009).

1.4.1.10 Chondroitin Sulfate

Chondroitin sulfate (CS) has been obtained from many marine organisms like whale, shark, squid, king crab, salmon, and sea cucumber. Also, it has been detected in marine invertebrates, such as Cnidaria, Polychaeta, and mollusks (Nandini et al. 2005). Among them, shark cartilage was the widely commercially approved source for nonmammalian CS, but its price has risen, and the ecological factors are the reasons why new innovations are anticipated in the years ahead (Im et al. 2009).

Structurally, CS decomposes from a basic disaccharide unit of hexosamine (D-galactosamine) and hexuronic acid (D-glucuronic acid) arranged in alternating unbranched sequences, which may bear sulfate ester substituents in a variety of positions. Variations in molecular weight, chain length, and sulfate substitution position vary per species, making the sequence diverse (Kinoshita et al. 2001). The CS chains have intriguing activities in central nervous system development, wound repair, infection, growth factor signaling, morphogenesis, and cell division, besides their traditional structural responsibilities (Sugahara et al. 2003). Practically, the CS may be removed by proteolytic digestion along with other GAGs and then separated from other GAG contaminants by organic solvent precipitation or enzymatic degradation of contaminant GAG species (Rocha et al. 2000).

1.4.1.11 Dermatan Sulfate

Marine dermatan sulfate (DS) has been isolated from ray skin, *Raja radula* (Ben Mansour et al. 2009); obtained from the clam, *Scapharca inaequivalvis* (Volpi and Maccari 2008); and found in the amphibian, *Bufo ictericus* (Pelli et al. 2007). Structurally, DS is composed of linear polysaccharides represented by a hexosamine, N-acetyl galactosamine (GalNAc), or glucuronic acid (GlcA) as disaccharide basic units. All DS components serve as potential biological response modifier since they act as (i) stabilizer, cofactor, and/or co-receptor for growth factors, cytokines, and chemokines; (ii) enzyme activity regulator; (iii) signaling molecules in response to cellular damage, like wounding and tumorigenesis; and (iv) target for microbial virulence factors for attaching, invasion, and immune system evasion (Trowbridge and Gallo 2002).

1.4.1.12 Heparan Sulfate

Heparan sulfate (HS) is a carbohydrate that belongs to the GAG family and is structurally similar to heparin. Several workers reported the isolation of HS from a numerous marine sources, such as mollusks, *Tapes philippinarum* (Cesaretti et al. 2004); crustacean, *Penaeus brasiliensis* (Saravanan and Shanmugam 2010); and invertebrates (Mourao and Pereira 1999) and algal heparinoids (de Azevedo et al. 2009). The HS is found on the cell surfaces or in the extracellular matrix of all mammalian organs and tissues in the form of proteoglycans, unlike heparin, which is just generated by connective tissue mast cells. Structurally, HS polysaccharide consists of continuing D-glucuronic acid or L-iduronic acid and D-glucosamine residues (Nagarajan et al. 2018).

Indeed, HS is excited in all mammalian tissues as a proteoglycan containing two or three HS chains that are tightly linked to cell surface or extracellular matrix proteins. Consequently, such poses HS for binding to a number of protein ligands and controls a wide range of biological activities, including developmental processes, angiogenesis, blood clotting, and tumor metastasis (Lindahl and Li 2009).

1.4.1.13 Keratan Sulfate

Keratan sulfate (KS) is present in some brachiopods, particularly in extracellular matrix of lophophores (Cole and Hall 2004). Also, it was detected in catfish, *Corydoras aeneus*, and loaches, *Acanthophthalmus semicinctus* and *Botia horae*. It is bounded to the protein core via an N-glycosyl linkage between N-acetyl-D-glucosamine and asparagines (Ito and Yamagata 1984; Ralphs and Benjamin 1992). Recently, a KS disaccharide [GlcNAc6S(β1–3)Galactose(β1-]-branched CS-E was identified from the clam *Mactra chinensis* (Higashi and Toida 2017). Structurally, the KS basic unit is a repeating N-acetylated lactosamine (Huckerby 2002). Commercially, the isolation of GAGs is carried out according to Maccari et al. (2015) via acetone defatting, proteolysis, collection of the GAGs, and fractionation of GAGs by anion-exchange chromatography and finally eliminating salts by desalting step.

1.4.1.14 Hyaluronic Acid

Hyaluronic acid (HA), also called hyaluronan, is a non-sulfated glycosaminoglycan with a major intercellular component of most connective tissues, such as cartilage, human eye vitreous, umbilical cord, and synovial fluid (Liao et al. 2005). It is also present in the umbilical cord and rooster comb (Pomin and Mulloy 2018). Mostly, HA is found in the marine cartilaginous fishes and in the vitreous humor of different fishes. Furthermore, the hyaluronan can be easily produced in large scales through microbial fermentation of streptococci. In addition to recombinant technology, commercial hyaluronan can be produced by extracting with water from the rooster comb, umbilical cord, synovial fluid, or vitreous humor and then precipitated with appropriate organic solvent (Liao et al. 2005).

Structurally, HA consisted of alternating disaccharide units of a-1,4-D-glucuronic acid and b-1,3-N-acetyl-D-glucosamine, linked by b (1R3) bonds (Pomin and Mulloy 2018). The polymers of HA possess extraordinarily wide-ranging biological functions depending on the size of the molecule (Tammi et al. 2002) as follows:

1. *In cartilage* – Aggregation centers for aggrecan, a big proteoglycan chondroitin sulfate, are formed by HA, which serves as an essential structural part of the matrix (Prehm 2002).
2. *In the skin* – HA functions as a scavenger of free radicals created by sunlight's UV radiation, which would otherwise cause oxidative stress in cells, potentially causing genetic damage (Juhlin 1997).
3. *In synovial fluid* – The high concentration of high molar weight HA provides necessary joint lubrication and acts as a shock absorber, reducing moving bone tension and reducing joint wear. However, as the viscosity of the HA decreases, its lubricant and shock-absorbing properties are compromised, resulting in impaired joint movement and pain (Soltes et al. 2006).

1.4.2 Bioceramics

Besides biopolymers, natural marine materials from sponges, corals, and nacres are an abundant source of inorganic materials significantly relevant for tissue replacement and regeneration. The context will focus on the marine calcium compounds (carbonates and phosphates) and silicates under this topic, while their relevance for biomedical applications will be explained below.

1.4.2.1 Hydroxyapatite

Hydroxyapatite (HAp) is typically prepared using a synthetic process with several benefits. However, many marine sources have also been used to extract HAp using different methods such as the conventional thermal calcination method, the alkaline hydrolysis process, and the polymer-assisted process (Venkatesan et al. 2010). The HAp has been detected to play an essential role in several bone-related applications, in particular, in the form of nanocrystals (Chesnutt et al. 2009). HAp has

considerable biocompatibility in bone tissue because of its chemical composition, which is similar to bone material (Sopyan et al. 2007).

1.4.2.2 Calcium Carbonates and Phosphates

Calcium-phosphorus compounds like HAp have a specific significance in the biomedical field since they are similar to the mineral elements of bones. The plentiful calcium carbonate may be the base material used to produce different calcium phosphates. While there are many sources of calcium carbonate, much focus has been drawn to coral skeletal carbonate, notably as substitute materials for orthopedics and dentistry. Corals have a unique architecture, namely, porosity, porous size, and interconnectivity of the pores. Calcium carbonate compounds (such as forms of aragonite or calcite) are found in numerous marine organisms (Laine et al 2008).

Actually, the characteristics of calcium-phosphorus compounds have been applied in the regeneration of bone tissue. In addition to microstructure, other characteristics, such as microstructural composition and mechanical properties, play a significant role in the in vivo activities of these biomaterials. Coral-derived materials have mostly been used in the form of granules and blocks for bone grafting, as well as scaffolding for bone tissue engineering (Kamenos et al. 2009). For instance, many routes to convert the calcium carbonate skeleton of red algae *Corallina officinalis* into calcium phosphates are described by Oliveira et al. (2007). By performing both thermal and chemical treatments, the possibility to obtain a calcium-phosphate material with HAp nanocrystallites rises up.

Furthermore, calcium phosphates, especially HAps, can be found in fish bones and seashells and can be obtained directly from marine resources. For example, Boutinguiza et al. (2011a) have described a laser-based approach for producing calcium-phosphate compounds in microparticulate form from fish bones. Using a continuous wave, HAp powder can be converted to nanoparticles, and various crystal forms can be created, with pulsed lasers promoting crystalline nanoparticle creation and continuous-wave lasers promoting amorphous nanoparticle development (Boutinguiza et al. 2011b).

1.4.2.3 Biosilica

Many aquatic species, including sponges, diatoms, radiolarians, and choanoflagellates, create biosilica, also known as biogenic silica, which is made of glassy amorphous silica. Demospongiae and Hexactinellida are the two kinds of sponges that have a silica skeleton, while Calcarea has a calcium carbonate skeleton (Schroder et al. 2008). One example of a silica skeleton of a sponge from Hexactinellida class is the impressive skeleton of *Euplectella aspergillum* species (Aizenberg et al. 2005). The silica skeleton, on the other hand, is made up of silica spicules, which are glassy spikes with an axial filament surrounded by hundreds of dense layers of hydrated silica (Venugopal 2009).

Silica can be extracted by soaking collected sponges in a 25% solution of sodium hypochlorite until all cellular material has been removed, then washing the residual material with water, and soaking it overnight in concentrated HNO3/H2SO4 (1:4).

The acid-insoluble substance that results is made up of silica spicules that have been cleansed with a silicate in filament (Brutchey and Morse 2008).

Sponge spicules have been shown to be highly flexible and durable, and they are used as electrical conductors, with the electrical charge presumably being transferred along collagen-formed channels, as deproteinized spicules have a lower electrical conductivity (Kubisz and Ehrlich 2007). Therefore, sponge spicules are fascinating structures that can be used to teach biomimetic design of a variety of devices, such as optical fibers, that can be manufactured at room temperature, demonstrating the advantages of biological synthesis (Woesz et al. 2006).

On the other hand, diatoms are a broad category of microalgae that are usually unicellular. The diatom's live component is contained within a silicon dioxide cage (Venugopal 2009). Frustules are exoskeletons comprised of silica nanoparticles that are formed into a highly structured structure with porous networks at various scales. Frustules are composite materials made up of silica nanoparticles packed with a matrix of carbohydrates and proteins. Their nanostructure is genetically determined and species-specific (Schroder et al. 2008).

Incredibly, the siliceous exoskeletons remain intact when cells die, giving morphological character to inorganic structures. In the seas, the inorganic elements sink to the ocean floor following cell death, reappearing to the surface as "diatomaceous earth" following millions of years of aging. Biosilica can be used as fresh diatomaceous silica, harvested from the field or prepared from cultivation to be low metal pollutants, suggesting that diatoms synthesize an almost pure silica matrix and are not needed for purification. Biosilica is thus considered for biomedical approaches, namely, bone substitution and regeneration strategies. For example, when human osteogenic sarcoma cells (SaOS-2) were grown on biosilica surfaces in the presence of b-glycerophosphate, they showed enhanced mineralization activity (Muller et al. 2009).

Moreover, Lopez-Alvarez et al. (2008) described the marine precursors for the biomorphic silicon carbide ceramics as a thin, tough, and high-strength material with predictable microstructure which is the resultant product. This material has been derived from the aquatic plant *Juncus maritimus*, since the morphology of the plant can be observed after pyrolysis and before and after silicone infiltration.

1.4.3 Marine Fatty Acids

Marine fish and shellfish contain good fatty acid composition and could bring many health benefits. The prevalence of daily marine fish consumption among rural and urban adults was 51% and 34%, respectively. As a result, it's critical to enhance understanding of the diverse nutrient contents of fish and shellfish species by providing full nutritional value information, particularly for fatty acid content linked to a variety of health impacts (Abd Aziz et al. 2013). Also, marine fish is a potent source for docosahexaenoic acids (DHA) and eicosapentaenoic acid (EPA). In addition, microalgae are used to make fatty acid or omega-3 fatty acid. Marine

fatty acids, especially EPA and DHA, have long been utilized to treat heart problems (Farzaneh-Far et al. 2010) and cancer (Patterson et al. 2011).

Commercially, the extraction of fatty acids is carried out according to Kinsella et al. (1977). However, the specimens of fish fillets are homogenized with a mixture of methanol and chloroform, which is filtered and then transferred to a separatory funnel to collect the lower phase and concentrated with a rotary evaporator. Lipid samples were converted to their corresponding methyl esters then concentrated to a fine volume for injection into the gas chromatograph. Quantitatively, the concentration of fatty acids is measured and expressed in mg/g of total lipids against tridecanoic acid methyl ester as an internal standard (Johnson and Saikia 2009).

1.4.4 Marine Pigments

Marine pigments are widely spread in marine algae and also in all living matters as fungi, invertebrates, and mammals. Not only do these pigments display the color, they are composed of various chemical components like terpenoids and melanins, which are found particularly in bacteria. The secondary metabolites of marine bacteria, especially colored species, show potential biological characteristics like antibiotics and anticancers. In such a manner, marine actinomycetes, *Pseudoalteromonas*, and cyanobacteria are widely investigated species (Delgado-Vargas et al. 2000).

Depending upon their pigment concentrations, the marine algae are divided as brown (Phaeophyceae), red (Rhodophyceae), and green algae (Chlorophyceae) (Khan et al. 2010). However, the main classes of algal pigments (chlorophylls, carotenoids, and phycobiliproteins) are as follows:

1. *Chlorophylls* – which are substituted tetrapyrrole with a centrally bound magnesium atom where the porphyrin tetrapyrrole is further esterified to a diterpene alcohol, phytol, to produce chlorophyll (Ferruzzi and Blakeslee 2007).
2. *Carotenoids* – which are linear polyenes that operate as light energy harvesters and antioxidants, inhibiting the formation of reactive oxygen species caused by light and air exposure (Ioannou and Roussis 2009). Moreover, carotenoids are divided into two kinds: carotenes, which are unsaturated hydrocarbons, and xanthophylls, which include one or more oxygen-containing functional groups (Sousa et al. 2006).
3. *Phycobiliproteins* – which are pigments that absorb energy in the visible range (450–650 nm) and are employed as an auxiliary or antenna pigment for photosynthetic light collection (Sousa et al. 2006). However, Table 1.1 shows some sources and bioactivities of these pigments.

Additionally, natural pigments have gotten a lot of interest among the useful compounds discovered in marine algae. Bioactivities like anticancer, antioxidant, anti-inflammatory, anti-obesity, antiangiogenic, etc. are among the many biological properties of these pigments (Pangestuti and Kim 2011).

Table 1.1 Marine-derived natural pigments and some of their potential bioactivity

Marine pigment	Main source	Bioactivity
Chlorophyll a	*Enteromorpha prolifra* *Fucus vesiculosus*	Antioxidant
β-Carotene	*Porphyra tenera*	Antimutagenic
Fucoxanthin	*Fucus serratus* *Undaria pinnatifida*	Antioxidant Anticancer
Phycoerythrin	*Aglaothamnion neglectum* *Gracilaria* sp.	Brightest fluorescent dye Labeling antibodies
Phycocyanin	*Aphanizomenon flos-aquae* *Spirulina* sp.	Anti-oxidation Anti-inflammation

1.4.5 Marine Toxins

Marine toxins usually consist of organic compounds that attract scientists to their inordinately high biological and biomedical activity. Depending on their categorization, marine toxins can be classified as alkaloids, steroids, peptides, or proteins, and their biological action can vary dramatically. Porifera, cnidarians, mollusks, echinoderms, vertebrates, sea snakes, and fish have all been identified as major marine toxin sources. Their production from marine animals is an important strategy. The use of marine creatures in their manufacturing is an essential approach (Gerssen et al. 2010).

Particularly, phycotoxins often accumulate in a variety of marine species, such as fish, crabs, and shellfish, such as mussels, oysters, scallops, and clams. Toxins accumulate in the digestive glands of shellfish, although they have no adverse impacts on the shellfish themselves. Clearly, consuming large amounts of infected shellfish by humans can result in severe consumer intoxication (Van Dolah and Ramsdell 2001).

In particular, different algal species produce marine toxins under certain conditions. These toxins may be then accumulated in the aforementioned species. A severe intoxication may occur when contaminated species of shellfish are consumed. The main detected toxins are amnesic shellfish poisoning (ASP), paralytic shellfish poisoning (PSP), diarrheal shellfish poisoning (DSP), azaspiracid shellfish poisoning (AZP), and neurological shellfish poisoning (NSP). These toxins are analyzed in mice or rats by way of bioassay. Subsequently, the recent methods for extracting many lipophilic marine toxins have been developed based on the functional tests such as the chemical assays and biochemical methods (Gerssen et al. 2010).

1.4.6 Microbially Marine Bioactive Substances

Certainly, the marine environment is a mostly untapped source for discovering novel microorganisms (bacteria, fungus, actinomycetes, cyanobacteria, microalgae, and diatoms) that are bioactive secondary metabolite makers. Significantly, Blunt et al. (2004) ordered the marine organisms in the environment descendingly as follows:

sponges (37%), coelenterates (21%), and microorganisms (18%), which are major sources of biomedical compounds, followed by algae (9%), echinoderms (6%), tunicates (6%), mollusks (2%), bryozoans (1%), etc. (Bhatnagar and Kim 2010).

Nonetheless, marine microorganisms have isolated a variety of biologically active substances of varying degrees of action, such as antimicrobial, antiviral, antitumor, anticancer, antiproliferative, cytotoxic, photoprotective, and antibiotic and antifouling properties. Some of these metabolites with high antibacterial and antifungal activities have been applied intensively as antibiotics and can be effective against infectious diseases such as HIV, multiple bacterial infection conditions (penicillin, cephalosporins, vancomycin, and streptomycin), or neuropsychiatric sequelae (Pandey 2019) (Fig. 1.3).

The general biophysical and biochemical properties and also the chemical structures of bioactive substances derived from marine microorganisms make them suitable for cosmeceutical and nutraceutical applications (Bhatnagar and Kim 2010). Indeed, many of these metabolites are widely used as antibiotics (e.g., penicillin, cephalosporins, streptomycin, and vancomycin) and may be effective against several infectious diseases like HIV-1, multiple bacterial infection conditions or neural tube defects, and neuropsychiatric sequelae (Berdy 2005).

Some others have also been used against several types of cancers (e.g., bleomycin, dactinomycin, doxorubicin, and staurosporine) and risk of coronary heart disease or may act as immunosuppressants (cyclosporin) to aid in organ transplantation (Ruiz et al. 2010). In addition to synthetic drugs, pharmaceutical companies now focus on natural products from marine microorganisms in most developed and developing countries (Bhatnagar and Kim 2010).

Moreover, polyhydroxyalkanoates, as biomedical materials, have the potential to replace the petrochemical plastics in surgical pins, sutures, staples, blood vessel replacements, bone replacements, and plates, medical implants, as well as drug delivery devices due to their highly effective biodegradability and biocompatibility. Microbes create a lot of PHA and ought to be researched more in this area (Khanna and Srivastava 2005).

Furthermore, it is known that many marine microorganisms create nanostructured particles with characteristics similar to chemically produced materials. The formation of magnetic nanoparticles by magnetotactic bacteria, the growth of silver nanoparticles inside the periplasmic space of *Pseudomonas stutzeri*, and the synthesis of palladium nanoparticles utilizing sulfate reduction bacteria are all examples of this (Pages et al. 2008). However, the periplasmic space and vesicles compartmentalization allow greater size control when nanoparticles are synthesized utilizing microorganisms. Controlling factors like pH, temperature, substrate concentration, and period of substrate exposure might help regulate the pace of intracellular particle production and hence the size of the nanoparticles to some extent (Gericke and Pinches 2006a, b).

Intracellular SNPs produced by *Idiomarina* sp. PR58–8, a marine bacterium, were shown to be extremely resistant to silver (Seshadri et al. 2012a, b). Microbes produced from mangrove *Escherichia coli*, *Aspergillus niger*, *Penicillium fellutanum*, and *Thraustochytrids* are capable of rapidly reducing silver ions in

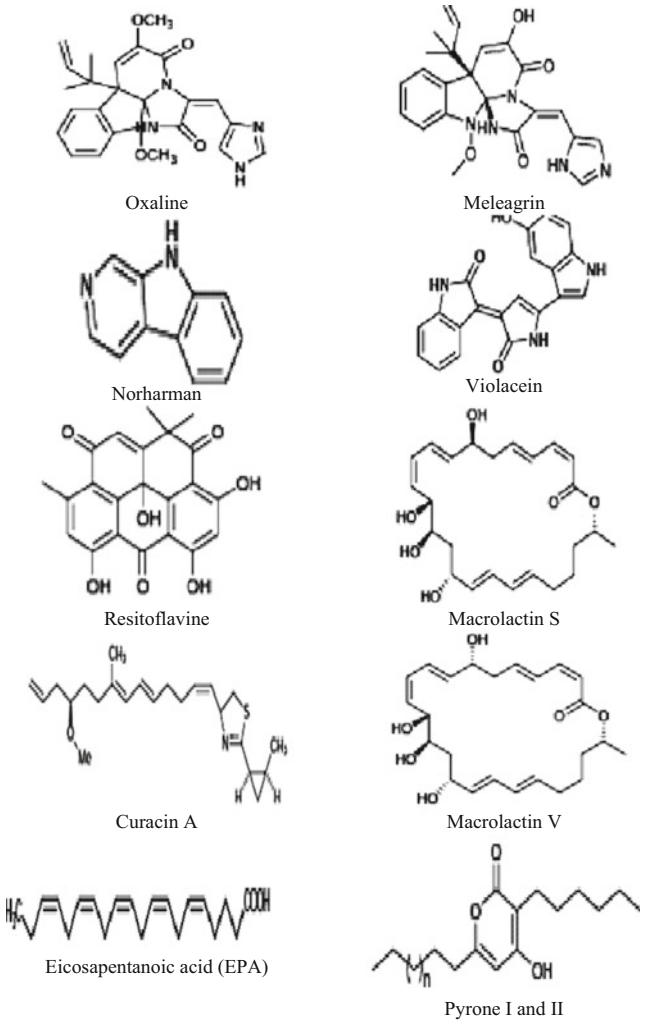


Fig. 1.3 A side of some potent marine microbial metabolites

antibacterial applications (Subramanian et al. 2010). Some mangrove-derived yeast species, such as *Pichia capsulata* and *Rhodosporidium diobovatum*, have been found to have the ability to synthesize nanoparticles, similar to these marine bacteria and fungi (Subramanian et al. 2010). The protein responsible for the reduction of silver ions and stability of silver nanoparticles is secreted by the marine cyanobacterium *Oscillatoria willei* (Ali et al. 2011). Afterward, Ali et al. (2012) used the marine cyanobacterium *Phormidium tenue* NTDM05 to synthesize cadmium sulfide nanoparticles.

1.5 Potential Applications of Marine Biomaterials

Due to unique properties and characteristics of marine organisms and their biomaterials, their numerous applications have been recently proposed for more than one field of life (Rahman 2016). Antiviral, antibacterial, antifungal, anticoagulant, antimalarial, antiprotozoal, antituberculosis, and agents have been identified from marine creatures, algae, fungus, and bacteria. Biomaterials are chemicals derived from marine creatures that have been discovered to be potential medicinal agents on several occasions (Schumacher et al. 2011).

Commercially, at least 14 biotechnology companies, mostly placed in North America and Europe, are known to be extensively engaged in product development and/or collaboration with research institutions in the search for new substances and compounds from deep-sea organisms and genetic resources both within and outside national jurisdiction. There are now six firms promoting products.

Despite the high costs of product development and commercialization, not more than 2% of candidates are clinically approved and produced, profits from a compound derived from a sponge to treat herpes have been estimated as US$50–100 million per year, and the estimated value of anticancer agents derived from marine organisms is up to US$1 billion per year (UNEP 2006).

1.5.1 Biological Applications

1.5.1.1 Production of Antibiotics

Antibiotics with novel structural classes must be discovered and developed for a variety of reasons. Resistance to existing antimicrobials is increasing, as is the toxicity of some of the currently available medicines, limiting their usage (Anand et al. 2019).

Just a small portion of the total number of living animals in marine habitats have been discovered and documented (Nweze et al. 2020). For instance, an antibacterial, coumarin-6-ol, 3,4-dihydro-4,4,5,7-tetramethyl, produced by *Streptomyces* VITAK1 showed inhibition to methicillin-resistant *Staphylococcus aureus* and other Gram-negative and Gram-positive pathogens (Abirami et al. 2015). A strain of *Streptomyces rochei* PM49 secretes sulfanyl cyslabdan-like product, which exhibits activity against MDR and ESBL-producing strains (Shanthi et al. 2015).

Recently, new compounds with anti-MRSA activity, paraphaeosphaeride D and berkleasmin F and A, were isolated from an artificial pond sediment fungus *Paraphaeosphaeria* sp. These compounds not only enhanced anti-MRSA activity (Suga et al. 2016). Furthermore, a fungus, *Pestalotia* sp., associated with the mangrove plant, *Heritiera fomes*, exhibited antimicrobial activities especially against MRSA strain (Nurunnabi et al. 2018).

1.5.1.2 Production of Antiviral Agents

The peak of antiviral pharmacology based on the natural products from seawater reached during 1999. Many marine polyketides, terpenes, compounds containing

nitrogen, and polysaccharides have demonstrated remarkable in vitro activity against pathogenic viruses (Uzair et al. 2011). Antiviral activity was screened against human immunodeficiency virus 1 (HIV-1), herpes simplex virus-2 (HSV-2), Junin virus (JV), poliovirus (PV), severe acute respiratory syndrome (SARS) virus, and measles virus and influenza virus. Antiviral chemicals, halichondrin B, homohalichondrin B, and isohomohalicondrin B, were obtained from the sponge *Lissodendoryx* sp. These chemicals have reduced tumor cell growth during in vivo and in vitro replication experiments. Viruses are the causatives of the most deadly diseases such as cancer, acquired immune deficiency syndrome (AIDS), and herpes simplex (Arshad et al. 2019). Polycitone A, an aromatic alkaloid identified from an ascidian *Polycitor* sp., functions as a superior inhibitor of HIV and retrovirus reverse transcriptase (Bailly 2015).

In a marine *Pseudomonas* sp., a glycosaminoglycan substance was discovered. This chemical has antiviral properties against influenza A and B viruses (Mayer and Hamann 2002). The topoisomerase enzyme of the poxvirus *Molluscum contagiosum* was inhibited by a sansalvamide antiviral chemical derived from a champignon, a fungus, *Fusarium* sp. (Hwang et al. 1999). In the influenza virus, calyceramides produced from the sea sponge *Discodermia calyx* inhibited neuraminidase (Nakao et al. 2001).

Other antiviral compounds are isolated from different marine fungi such as *Aspergillus* sp., *Penicillium* sp., *Stachybotrys* sp., *Cladosporium* sp., and *Neosartorya* sp. Among them, one compound revealed marked inhibitory activity against HSV, and others showed a powerful anti-influenza virus activity (Moghadamtousi et al. 2015).

1.5.1.3 Production of Antioxidant Agents

The toxicity of several synthetic antioxidants can exhibit many health problems in human. Therefore, several researchers have paid attention to many safe natural antioxidants to be used without any toxicity in human. Marine bacteria and microalgae, on the other hand, are the primary creators of important compounds such as antioxidant enzymes (e.g., catalase and superoxide dismutase) and antioxidant substances (e.g., exopolysaccharides, carotenoids, and bioactive peptides) with valuable biological applications (Nolan et al. 2015). In addition, using marine biomaterials including peptides, chitosan and chitosan derivatives, as well as sulfated polysaccharides like fucoidan and laminar, numerous researchers examined antioxidant properties (Kim and Venkatesan 2013).

1.5.1.4 Production of Anti-Inflammatory Agents

Currently, certain classes of drugs with anti-inflammatory activity like aspirin, nonsteroidal anti-inflammatory drugs (NSAIDs), and corticosteroids are applied in the clinic with the development of the synthetic drug formulation (Kim et al. 2016). All of the therapies, however, after long-term medication with high dose, can cause quite harmful side effects to human. Many new secondary metabolites produced from marine-derived fungus have been identified, all of which have significant anti-inflammatory properties. Marine fungal compounds have gotten a lot of interest

lately because of their unique mode of action, and they've become one of the most promising areas for anti-inflammatory medication research. However, over 50 of 133 substances belonging to 5 different chemical families, including alkaloids, terpenoids, polyketides, peptides, and other substantially anti-inflammatory properties, have been discovered in the previous 2 decades (Xu et al. 2019).

1.5.1.5 Production of Anticancer Agents

Drugs used to treat cancer are often very toxic, killing both cancerous and non-cancerous cells. By combining immunotherapy with cuttlefish, *Sepiella maindroni* and *S. rochebruns*, Liu et al. (2015) created a more powerful cancer treatment regimen.

Cuttlefish ink is largely made up of melanin and proteoglycan, and it is a byproduct of the processing of marine products. Because of its anticancer, immunomodulatory, and hemostatic properties, cuttlefish ink is frequently utilized in folklore Chinese medicine. Sulfated polysaccharides/glycoproteins have been shown to have anticancer properties (Kim and Venkatesan 2013). Over 200 polysaccharides/glycoconjugates have been isolated from the well-known traditional Chinese medicine, *Ganoderma lucidum* (Cor et al. 2018).

1.5.2 Biomedical Applications

1.5.2.1 In Process of Drug Delivery

Drug delivery systems in the future can be described as means for introducing therapeutic substances into the body to aid or improve tissue function in various organs. Natural polymers derived from marine resources have gained popularity in recent years due to their abundance and biological activity when compared to polymers derived from other sources (Rizvi and Saleh 2018).

To encapsulate or load the desired amount of protein, alginate microspheres are used in order to protect the protein's activities, transport it to the required locations, and control the protein's release kinetics. Layer-by-layer deposition methods were used to coat alginate microspheres with *Bombyx mori* silk fibroin, which produced a mechanically stable shell and a diffusion barrier for the encapsulated proteins (Kim 2015).

1.5.2.2 In Process of Gene Delivery

Gene therapy, which involves restoring missing genes, replacing faulty genes, or suppressing gene expression to treat inherited and acquired illnesses, is a revolutionary kind of molecular medicine that has a significant influence on human health in the next generation (Goncalves and Paiva 2017). In gene therapy, one of the big hurdles is the lack of a suitable gene carrier (Scheller and Krebsbach 2009).

The use of marine polymeric materials such as chitosan, alginate, and pullulan as gene delivery carriers has been considered. Because of their biocompatibility, low immunogenicity, and low toxicity, they are attractive nonviral vectors. Furthermore, they are simple to modify for the suggested applications while maintaining desired

physicochemical and physiological characteristics. With positive charges, they are also biodegradable, biocompatible, less toxic, and less immunogenic (Huh et al. 2017).

The transfection effectiveness of chitosan/DNA complexes or gene silencing of chitosan/siRNA complexes is affected by a number of factors including pH, molecular weight (MW), and degree of deacetylation of chitosan, N/P ratio, serum, and cell type. Also, DNA or siRNA complexation and cellular uptake of the polyplexes at the extracellular environment, release of DNA or siRNA from endosomes in the cytoplasm, release of DNA or siRNA from the chitosan carrier, and DNA transfer into the nucleus at the intracellular environment are also steps in gene transfection or gene silencing. Particularly, the delayed release of DNA from chitosan/DNA complexes from endosomes into the cytoplasm is one of the major problems with poor DNA transfection efficiency or siRNA gene silencing by chitosan (Cao et al. 2019).

1.5.2.3 In Engineering of Tissue Repairing

Tissue engineering's major objective is to use a sensible mix of cells, biomimetic matrices, biological signals, and biophysical cues to replace or repair the normal biological activities of tissues or organs. By providing particular cell recognition sites and signaling molecules in a structure, polymeric scaffolds are meant to actively regulate cell activities and promote tissue growth in a temporal and spatial manner (Chen and Liu 2016).

Due to its strong similarity to glucosaminoglycans, a component of the extracellular matrix that interacts with collagen fibers and therefore promotes cell adhesion, chitosan is a suitable material for bone tissue creation. Chitosan may be converted into various structural geometries for regenerative applications, just as textiles, films, and sponges (Dey et al. 2016). To improve its osteoinductive capabilities, it can be transformed into lyophilized tridimensional scaffolds and mixed with ceramic materials (Rinaudo 2006). Chitosan may produce high porosity networks, which provide a perfect internal architecture for cell adhesion and proliferation, in addition to its outstanding biocompatibility (Rashid et al. 2014).

Because of its antibacterial characteristics, the use of chitosan for guided bone regeneration has gotten a lot of interest (Aguilar et al. 2019). For example, Lee et al. (2009) synthesize a guided bone regeneration membrane by combining hybridized chitosan with silica xerogel via sol-gel. The resulting membrane has the normal features of any membrane, including flexibility and bioactive capabilities that make it appropriate for other molecules. The hybrid's apatite nucleation ability, cellular response, and mechanical characteristics were all substantially linked with the membrane's bone regeneration capabilities. The utilization of marine structures/species as a template for developing synthetic skin tissue is proposed as a potential method (Lim et al. 2019).

In terms of biological, physical, and mechanical characteristics, the ideal synthetic skin substitute should be quite comparable to real skin. Marine organism-derived collagen scaffolds have been found to be extremely absorbent, elastic, and resistant to high temperature and bacterial assault in studies, indicating that they

have a lot of potential in therapeutic applications. When implanted in Sprague Dawley rats, biomimetic tilapia collagen nanofibers also encouraged fast skin regeneration (Halim et al. 2010). Furthermore, research has focused on different gelatin-based composites scaffolds and microspheres to increase hepatic cell activities, such as chitosan/gelatin and silk fibroin/gelatin. By using 3D bioprinting microtechnology tools and biomaterials, to investigate hepatic cell activities and inquire to mimic human illnesses, it is now possible to manufacture 2D and 3D hepatic microenvironments (Nikolova and Chavali 2019).

1.5.3 Pharmaceutical Applications

More innovative pharmaceutical and cosmetic goods using chemicals derived from natural resources have been developed as a result of a global trend for products that are deemed healthful, environmentally sustainable, and ecologically friendly (Amberg and Fogarassy 2019). So, many marine compounds have a great potential as cosmeceuticals or nutricosmetics due to their antioxidant, anti-inflammatory, anti-allergic, antiaging, anti-wrinkle, anti-tyrosinase, and MMP inhibitory activities as well as UV protection. Alkaloids, peptides, proteins, lipids, mycosporines, MAAs, glycosides, and isoprenoids are just a few of the chemicals produced from marine bacteria that have photoprotective, antiaging, antibacterial, antioxidant, and moisturizing properties (Alves et al. 2020).

Among the bioactive substances with the antiaging activity of the marine origin, exopolysaccharides (EPSs) are one of the most widely used cosmeceutical products (Guillerme et al. 2017). Deepsane, an exopolysaccharide derived from the marine bacterium *Alteromonas macleodii*, is commercially available under the name Abyssine® for soothing and reducing the irritation of sensitive skin against chemical, mechanical, and UVB aggression (Le Costaouëc et al. 2012). Antiaging products contain a combination of EPSs from *Pseudoalteromonas* sp. isolated from Antarctic waters, which boost collagen I production and improve skin structural characteristics (Martins et al. 2014). A deep-sea hydrothermal vent marine bacterium, *Vibrio diabolicus*, produces an EPS-HE 800 (Shindo et al. 2007).

Astaxanthin pigment is also produced by some marine-derived bacteria as *Paracoccus* sp. (Lee et al. 2004) and *Agrobacterium* sp. (Yokoyama and Miki 1995). Methylene chloride, produced by new marine *Pseudomonas* sp., can reduce the pigmentation of human melanocytes and cultured skin cells by inhibiting the expression of tyrosinase (Kang et al. 2011). The anti-tyrosinase activity of N-acyldehydrotyrosine analogues, thalassotalic acids derived from the marine bacteria *Thalassotalea* sp., has been detected (Bownik and Stpniewska 2016). Ectoine was discovered in *Ectothiorhodospira halochloris* and Actinobacteridae when they were exposed to high salt concentrations. This compound improves the hydration of the cell surface (Kunte et al. 2014).

Interestingly, some biosurfactants derived from marine microorganisms, such as mannosylerythritol, rhamnolipids, and sophorolipids, have been used in the cosmetic industry because of their emulsifying, solubilizing, wetting, foaming, and dispersing

properties, which can not only improve the solubilization of hydrophobic ingredients in products but also facilitate their delivery through the skin barrier (Fenibo et al. 2019), and they have low irritancy to the skin. Thus, it is ideal for the anti-wrinkle formulations (Varvaresou and Iakovou 2015).

In addition, algal alginate's traditional uses in pharmaceutics include thickening, gel-forming, and stabilizing agents. Alginate hydrogels containing poly (caprolactone), carbon nanotubes, and chitosan have been used to integrate a variety of medicines (Martău et al. 2019). Furthermore, significant findings have verified the antioxidant activity of fucoidan's sulfate contents (Wang et al. 2019), and there is good evidence for its function in wound healing by boosting fibroblast repopulation and angiogenesis (Savari et al. 2019).

Furthermore, microalgae such as *Chlorella*, *Spirulina*, *Dunaliella*, and *Odontella* species are rich sources of several bioactive compounds with potential applications as nutraceuticals and cosmeceuticals (Cotas et al. 2020). Microalgal cosmeceuticals are of great interest as some of them synthesize substances that absorb UV radiation, which can prevent dermal ECM deterioration, wrinkles, laxity, coarseness, and mottled pigmentation of the skin. For example, the cyanobacterial sunscreen pigment scytonemin absorbs UVA/UVB radiation more efficiently than a commercial formulation (Alves et al. 2020). Several cyanobacteria, including *Nostoc* sp., *Calothrix crustacean*, and *Chlorogloeopsis* sp., generate scytonemin (Singh et al. 2017). The halotolerant microalga *Dunaliella salina* may produce more than 10% of its dry weight from carotene, which is considered as another UV protection pigment (Fu et al. 2013). Astaxanthin can help skin health by affecting several stages of the oxidative damage cascade and reducing inflammatory mediators (Novoveská et al. 2019).

1.5.4 Food and Nutritional Applications

Bioactive substances (proteins, peptides, polysaccharides, fatty acids, polyphenols, probiotics, enzymes, vitamins, and minerals) with nutraceutical uses in the food and supplement sectors are found in and derived from marine environments. In the marine bioprocessing sector, there is also a lot of potential to convert and use most marine food items and byproducts as important functional products. Also, several nutrient-derived compounds such as chitooligosaccharides, flavonoids, polyphenols, and fatty acids are able to inhibit the activation and expression of matrix metalloproteinases (Suleria et al. 2015). So, these compounds could have a strong potential for the development of nutricosmetic products. However, enzymatic hydrolysis of marine foods permits the production of functional components such bioactive peptides and chitooligosaccharides (Zhang et al. 2012).

Lipase, chitinolytic enzymes, polyphenol oxidase (catecholase, tyrosinase, cresolase, polyphenols, catechol oxidase, etc.), and transglutaminase are enzymes originating from marine sources, and red algal enzymes have been implicated in the starch breakdown pathway (e.g., α-1,4-glucanase). Proteolytic enzymes from

microorganisms, plants, and animals may also be utilized in the production of marine food hydrol. In addition, proteolytic enzymes from microorganisms, plants, and animals may be utilized in the hydrolysis of marine foods to produce bioactive peptides and chitooligosaccharide derivatives (Razzaq et al. 2019).

Marine proteins of fish, crabs, mollusks, extremophiles like *Dunaliella*, and algae all have unique biochemical and biological characteristics. Collagen, gelatin, and albumin, for instance, are typical marine proteins that are enzymatically digested to produce bioactive peptides that can be utilized as nutraceuticals. Also, marine protein protamine is used in the food industry as a natural antibacterial preservative (Suleria et al. 2015).

Marine polysaccharides, primarily carrageenans, fucoidans, and alginates, offer a variety of biological activities that enhance their biological characteristics, allowing them to be used in nutraceuticals, especially in the dairy sector (Tanna and Mishra 2019). Seaweeds have extra health advantages since they are high in vitamins and minerals such as iron, iodine, manganese, and zinc. Some types of seaweed could be used as natural sources of iodine (Wells et al. 2017).

Many marine fish and algae species have also been discovered as rich sources of polyunsaturated fatty acids (PUFAs), particularly ω-3 and ω-6 fatty acids. The high amount of PUFAs in marine-derived foods makes them more useful as nutraceuticals in the food business, giving a variety of health advantages such as improved vision and neurodevelopment, relief from hypertension and arthritis, and weight loss (Ashraf et al. 2020).

1.5.5 Valuable Industrial Applications

Among biomaterials produced from the sea, polysaccharides are widely employed in a variety of sectors, including agriculture, wastewater treatment, paper manufacturing, etc. (Kim and Venkatesa 2013). Marine enzymes with novel and exciting catalytic activity have recently been brought to biocatalysis applications (Birolli et al. 2019). Proteases, a very significant industrial-type enzyme, are employed in the processing of leather, food, and detergents, to name a few examples (Kim 2015).

1.6 Remarkable Conclusions

The current chapter is an attempt to review and focus on the latest investigations and critical points in this research. Also, it showcases the tremendous efficiency of marine biomaterials and various bioactive byproducts. Also, it deals with some effective and new methods for producing marine biomaterials from different resources. Effort should be made for more comprehensive approach to investigate if the marine organisms, especially microbes, have opportunities in the field of biomaterials technology. Moreover, the present chapter illustrates the unique

features and properties of biomaterials, which qualify them for many future applications in several fields of life.

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Crosslinked Marine Polysaccharides for Delivery of Therapeutics

2

C. E. Iurciuc-Tincu, L. Ochiuz, M. Popa, and L. I. Atanase

Abstract

Marine biomaterials can be grouped into three broad categories: polysaccharides, proteins, and lipids. Among these, polysaccharides from both plants and marine animals are of the greatest interest. This is explainable—despite the fact that the basic structural unit is the glycosidic cycle—by the presence of various functional groups, with high chemical reactivity even in moderate conditions, which allow relatively easy chemical modifications, through biological properties such as biocompatibility, biodegradability, and anti-inflammatory activity as well as adhesive and, in some cases, antimicrobial activity. This chapter aims to present the different possibilities of crosslinking the polysaccharides of marine origin, its organization being made on the basis of the type of crosslinking method. Although, over time, numerous polysaccharides have been isolated from marine plant and animal organisms, at least so far some of them have not found their use

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in obtaining hydrogels. Therefore, all types of hydrogel formulations as drug delivery systems, studied in literature in the last decade and based on polysaccharides, such as chitosan, chitin, k-carrageenan, hyaluronic acid, alginic acid/alginates, agar/agarose, and fucoidan, will be discussed in this chapter.

Keywords

Marine polysaccharides · Drug delivery systems · Chitosan · K-carrageenan · Alginate · Agar

2.1 Introduction

Life appeared in the marine environment billions of years ago. Changes over time—temperature, radiation, pressure, pH, etc.—imposed the continuous evolution of marine species to adapt to those conditions, creating an extraordinary biodiversity. Blue technology, which refers to the exploration and exploitation of existing resources in various marine organisms, is constantly expanding and contributes significantly to the use of marine biological resources in many areas of daily life. Starting from this practically inexhaustible source of raw materials, scientists have developed materials with numerous applications in human health, agriculture energy, aquaculture, fine chemicals, pharmaceuticals, food, cosmetics, and environmental sectors including biosensing and bioremediation.

Marine biomaterials can be grouped into three broad categories: polysaccharides, proteins, and lipids. Among these, polysaccharides from both plants and marine animals are of the greatest interest. This is explainable—despite the fact that the basic structural unit is the glycosidic cycle—by the presence of various functional groups, with high chemical reactivity even in moderate conditions, which allow relatively easy chemical modifications, through biological properties such as biocompatibility, biodegradability, and anti-inflammatory activity as well as adhesive and, in some cases, antimicrobial activity.

Polysaccharides are defined as polymeric carbohydrate structures formed by repeated monosaccharide units that are attached to each other by glycosidic bonds. Despite their large bioavailability, polysaccharides are still underexploited in bio-applications. Among these biomedical applications, drug delivery systems and tissue engineering are important. The formulation of the polysaccharides is varied, including films, foams, inserts, implants, micro-/nanoparticles, micro-/nanocapsules, etc. From a structural point of view, regardless of their formulation, all these systems are made up of three-dimensional networks, in which the connection of macromolecular chains is made by chemical, physical, or combined bonds. Given the strong hydrophilic character of the constituent polysaccharides, the materials have an obvious hydrogel character.

This chapter aims to present the different possibilities of crosslinking the polysaccharides of marine origin, its organization being made on the basis of the type of crosslinking method. Although, over time, numerous polysaccharides have

been isolated from marine plant and animal organisms, at least so far some of them have not found their use in obtaining hydrogels. Therefore, all types of hydrogel formulations as drug delivery systems, studied in literature in the last decade and based on polysaccharides, such as chitosan, chitin, k-carrageenan, hyaluronic acid, alginic acid/alginates, agar/agarose, and fucoidan, will be discussed in this chapter.

2.2 Chemically Crosslinked Hydrogels

The realization of transverse bridges between the linear chains of a polymer can be done by chemical bonds, which in turn can be, in principle, covalent, ionic, coordinative, and also their combinations.

2.2.1 Crosslinking by Covalent Chemical Bondings

The covalent chemical bonds that ensure the formation of the network starting from linear polymers can be created by several processes: the use of *bifunctional crosslinking agents* with groups complementary to those of linear polymers; *grafting/crosslinking by polymerization* of previously obtained polysaccharide macromers; and *grafting/crosslinking by creating macroradicals* on the polymer chain.

2.2.1.1 Crosslinking by Functional Groups Reaction

It is well demonstrated that hydrogels prepared by chemical crosslinking are characterized by a greater stability, longer durability, and higher mechanical properties (tensile, shear, bending, etc.), compared with physically crosslinked hydrogels. These crosslinked hydrogels are generally obtained by a chemical reaction between two functional groups of the polymers, such as -COOH, -OH, $-NH_2$, etc., with crosslinking agents, such as aldehydes [glutaraldehyde (GA), adipic acid dihydrazide, etc.], *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide (EDC), or N-hydroxysuccinimide (NHS). In this section, the literature data from the last decade concerning the preparation, characterization, and potential applications of different types of covalent crosslinked hydrogels (films, beads, and micro-/nanoparticles) will be discussed.

Crosslinked sodium alginate (SA) films were prepared in the presence of GA and glycerol and loaded with diclofenac diethylamine, as model drug (Kulkarni and Wagh 2010). As expected, both the water uptake and drug release rate were dependent on the crosslinking density and films thickness. These flexible films were suitable and safe for transdermal drug delivery. The same research group prepared also interpenetrated polymer networks (IPN) matrices based on SA and carrageenan for the loading and release of a drug-resin complex based on propranolol HCl and Indion 254 (Kulkarni et al. 2011). The authors have demonstrated that the drug release was controlled by both the ion exchange resin and by the crosslinking degree. More recently, the same research group loaded the previous

drug-resin complex in IPN hydrogels tablets based on SA and tamarind seed polysaccharide (Kulkarni et al. 2013). A dual pH and thermo-responsive porous hydrogel was prepared from SA, poly(N-acryloylglycine methyl ester), and poly (acryloylglycine ethyl ester) in the presence of N,N'-methylenebisacrylamide (MBA) (Ma et al. 2013). Moreover, $CaCO_3$ particles were added and reacted with dilute HCl in order to produce CO_2 and $CaCl_2$, which acted further as an ionic crosslinking agent for SA. Caffeine was loaded as a model drug, and a higher cumulative release was observed at pH 7.4 and 37 °C than at room temperature and pH 1.2. Another interesting type of hydrogel films was obtained by an in situ crosslinking between oxidized SA and N-maleoyl-CS. These hydrogels were loaded with metronidazole and the highest drug entrapment efficiency was 99.2% (Pasaribu et al. 2019). No significant difference was observed between the cumulative drug releases, after 12 h, at pH 1.2 and 7.4. Very recently, magnetic hydrogels based on alginate, gelatin, and Fe_3O_4 NPs were loaded with doxorubicin (Dox) (Jahanban-Esfahlan et al. 2020). Drug encapsulation and drug loading efficiencies were determined to be 59 and 47%, respectively. The loading of Dox led to a decrease of the ZP values from −17.2 to −8.1 mV. This hybrid hydrogel exhibited a pH-sensitive behavior with a higher drug release rate at pH 4 than at pH 7.4 and 37 °C.

pH and temperature-responsive beads, based on alginate-graft-PNIPAM and crosslinked with GA, were loaded with indomethacin (IM) (Işiklan and Kücükbalci 2012). The obtained beads were spherical in shape with a porous surface. The release rate of IM was faster at pH 7.4 than at 1.2. Moreover, the drug release was influenced by the grafting density, drug/polymer ratio, and crosslinking density. In another study, insulin-loaded alginate/chitosan (CS) gel beads, crosslinked with GA, were obtained and studied for oral administration (Tahtat et al. 2013). The increase of the CS amount led to a decrease of the cumulative drug release during 6 h. Beads based on SA-graft-poly(N-isopropylacrylamide) (PNIPAM) and poly(acrylic acid) (PAA) were also prepared in the presence of GA and studied for the loading and release of IM (Işiklan and Kücükbalci 2016). Due to the presence of synthetic polymers, these beads were sensitive to both pH and temperature. The cumulative drug release at pH 7.4 and 37 °C was higher than at pH 1.2 and 25 °C. The drug release mechanism followed non-Fickian or Case II transport, depending on the bead structure.

Microcapsules based on alginate or pectin and gelatin, crosslinked with GA in the presence of sodium carboxymethyl cellulose, were loaded with three drugs, such as metronidazole hydrochloride, diclofenac sodium, and IM (Saravanan and Rao 2010). The highest encapsulation efficiency was obtained for IM. The microparticles formed by the system alginate/gelatin were characterized by a smaller average size than those of pectin/gelatin. Moreover, a lesser aggregation and easy dispersion was also noticed for the alginate-based system. Microspheres based on alginate and hyaluronate were prepared by crosslinking with GA and loaded with exenatide, at a loading efficiency of 71.4% (Zhang et al. 2014). The size of these microspheres was increased with the crosslinking duration. The drug release was complete after 7 h at both pH 1.2 and 7.4. Very recently, He et al. (2020) obtained reduction-responsive alginate-based microcapsules via a sonochemical method. Both oleic acid modified Fe_3O_4 NPs and coumarin 6 were simultaneously loaded in these

microparticles which had an optimum size of 1.5 μm and were obtained under 400 W ultrasonication for 8 min in the presence of 0.8% thiolated alginate. The cumulative release increased from around 10% after 48 h at 37 °C to 80% in the presence of 10 mM GSH. In order to obtain a controlled and sustained drug release, a hybrid hydrogel was prepared with tetracycline HCl-loaded gelatin microspheres dispersed in oxidized alginate and carboxymethyl chitosan (CMCS) hydrogel matrix (Chen et al. 2017). By increasing the amount of loaded microspheres, the swelling degree decreased but the mechanical properties increased. The optimal formulation had a concentration of 30 mg/ml gelatin-based microspheres. A sustained drug release was noticed owing to the double microspheres/hydrogel obstacle. The possible application of this formulation is wound healing as this hybrid hydrogel had an excellent antibacterial effect.

Nanoparticles (NPs) based on alginate and gelatin crosslinked with GA were used for the loading and release of Dox (Lee et al. 2014). An average size of 489.5 nm was determined by DLS and the drug release was controlled for over 2 weeks. In another study, Dox was conjugated on pegylated oxidized alginate NPs, crosslinked with fluorescent carbon dots in order to prepare tumor theranostic platforms (Jia et al. 2016). The obtained NPs had an average size of 26 nm and a Dox amount of 0.2532 mg/mg. The pH trigged the release of Dox in the tumor environment without a burst release. Moreover, the fluorescence was an asset for imaging-guided cellular drug delivery. Alginate-cysteine conjugate was used for the preparation of tamoxifen (TMX)-loaded nanoparticles (NPs) (Martinez et al. 2012). The size of these NPs was smaller than 500 nm and the zeta potential (ZP) values were in the range of −7 to −37 mV. During 24 h only 50% of the TMX was released in PBS at pH 7.4 and 37 °C, in the presence of an anionic surfactant (sodium dodecyl sulfate). The unloaded NPs were not cytotoxic, whereas the TMX-loaded NPs led to an effective elimination of the carcinoma cells. The same research group had also studied the TMX-loaded NPs based on thiolated alginate and disulfide bond reduced albumin (Martinez et al. 2011). SA nanospheres, obtained by self-assembly and crosslinking with disulfide bonds, were loaded with 5-aminosalicylic acid (5-ASA) for colon-specific drug delivery (Chang et al. 2012). The size decreased from 203 to 137 nm as the concentration of the SH groups, and thus the crosslinking density, increased. A similar trend was noticed for the ZP values which ranged from −25.7 to −20.3 mV. In the presence of 10 mM glutathione (GSH), the sizes also increased. A cumulative drug release of around 25% was noticed after 15 h, whereas in the presence of GSH, this value increased up to 85%. More recently, SA-based NPs were crosslinked with GA and loaded with zidovudine, an antiviral drug (Joshy et al. 2017). The obtained NPs, stabilized with Pluronic 68, had an average size of 432 nm. The drug release was studied at pH 7.4 and a cumulative drug release of around 55% was noticed during 24 h.

Paclitaxel (PCX) was loaded in a series of self-assembled cysteamine-based disulfide crosslinked SA nanospheres in order to control the delivery to colonic cancer cells (Ayub et al. 2019). A pH-sensitive swelling behavior was noticed in the pH range of 1 to 7. The average sizes of different drug-free samples ranged between 113.0 and 291.9 nm, whereas the drug loading led to an increase of these sizes. The

stability of these nanospheres was assured by the high values of the ZP, in the range of −30 to −52 mV. Moreover, the size increased by 33% after 7 days of incubation with GSH. Furthermore, the drug release in simulated gastrointestinal media was also affected by the presence of GSH. Fluorescent microscopy demonstrated that more than 70% of the nanospheres were internalized into HT-29 cells. The disulfide bonds procedure was also used for the preparation of crosslinked SA NPs by air oxidation starting from thiolated SA (Chiu et al. 2020). Fluorescein-labelled wheat germ agglutinin was conjugated to the surface of these docetaxel (DTX)-loaded NPs. A mean diameter of 289 nm and a ZP value of −2.2 mV were determined. A cumulative drug release of 48.6% at 37 °C, in the presence of GSH, was observed in simulated gastrointestinal media, and it was demonstrated that the drug release fits the Higuchi model which means that DTX is released by a diffusion and dissolution mechanism. Moreover, a cellular uptake efficiency of 29.9% was noticed by fluorescence microscopy after 24 h of incubation.

Chitosan (CS)-based hydrogel films were obtained by a basic method of crosslinking in the presence of GA (Cojocariu et al. 2011). Different active principles were loaded in these hydrogels and the drug release was investigated at pH 2.2. As expected, it appeared that both the characteristics of the matrix and drug's properties have an important influence on release rate. Genipin was used as crosslinking agent for the preparation of lidocaine-loaded catechol-functionalized CS hydrogels (Xu et al. 2015). The presence of catechol groups significantly increased the mucoadhesion both in vitro and in vivo. In another study, the same crosslinking agent was used for the preparation of semi-IPN based on CS and bacterial cellulose (BC) (Arikibe et al. 2019). These hydrogels were loaded with quetiapine fumarate (QF), which is an antipsychotic drug, and it appeared to be pH-sensitive. The swelling degree increased at high CS ratio and low pH value. On the contrary, at high pH value, swelling degree increased by rising the BC ratio. A controlled release of QF, following the Higuchi model, at different pH values (1; 4.5 and 6.8) was noticed. 3-Aminopropyltriethoxysilane was used as crosslinking agent for the preparation of CS/poly(vinylpyrrolidone)-based hydrogels for the controlled release of cefixime in gastric pH medium (Ata et al. 2020). Swelling degree was studied as a function of pH and the maximal value was noticed at pH 2. The drug release in simulated gastric fluid followed the Higuchi model. Bovine serum albumin (BSA) was loaded in a pH-sensitive hydrogel based on carboxymethyl CS sodium salt and sodium alginate which were crosslinked with N-ethyl-N′-(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydroxysuccinimide (NHS) (Xie et al. 2019). The swelling degree was studied at different pH values and the drug release was higher than 90% at pH 7.4. N-Succinyl-CS and oxidized alginate were crosslinked via the Schiff base reaction (Xing et al. 2019). In this hydrogel matrix were incorporated BSA-loaded microspheres based on sodium alginate ionically crosslinked with Ca^{2+}. As expected, the BSA release rate was slower from the hybrid hydrogels films than from microspheres. More recently, a dual UV and pH-responsive CS-based hydrogel was prepared, using a photo-cleavable crosslinking agent 4-formylphenyl 4-((4-formylphenoxy)methyl)-3-nitrobenzoate,

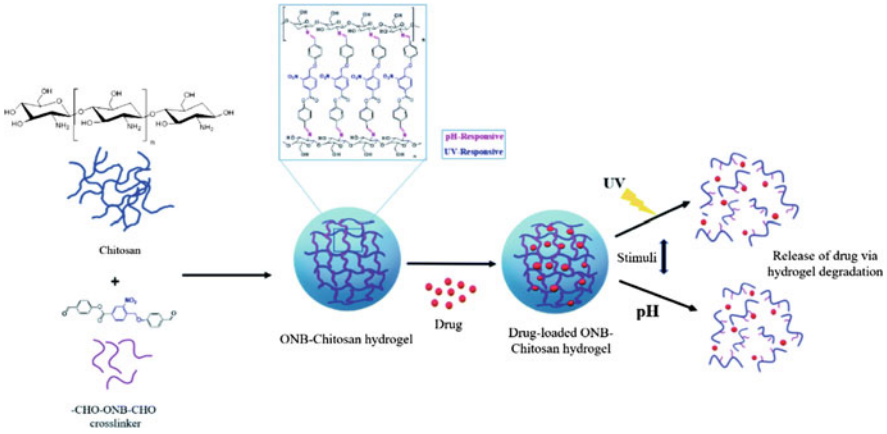

Fig. 2.1 Schematic representation of photo-cleavable and pH-responsive hydrogel proposed for drug delivery applications (Nisar et al. 2020; Reproduced by permission of The Royal Society of Chemistry)

for the loading of Dox HCl (Nisar et al. 2020). The schematic representation is provided in Fig. 2.1.

The highest swelling degree and therefore the highest drug release rate were noticed at pH 5.7 which is similar to the environment of cancerous tissues.

CS was combined with starch and crosslinked with both GA and sodium hexametaphosphate (SHMP) in order to obtain beads for the encapsulation of chlorpheniramine maleate (Kumari et al. 2016). The optimum conditions in order to achieve a maximum drug release were 60% CS, 40% starch, 10% SHMP, 15% GA, and 6.24 h drug release time at both pH 2.2 and 7.4.

Simple CS microspheres, crosslinked with GA, were loaded with famotidine by the classical emulsification technique (Ramachandran et al. 2011). The highest encapsulation efficiency was 73%, whereas the maximum cumulative drug release was 85.6% at 37 °C after 2 h at pH 2 followed by a release duration of 20 h at pH 6.8. Non-coated and pectin-coated curcumin-loaded CS microparticles were obtained by crosslinking with GA (Hwang and Shin 2018). Pectin was ionically crosslinked in the presence of Mg^{2+} or Ca^{2+} ions. The drug release was investigated at both pH 1.2 and 6.8. A slow curcumin release was noticed for the pectin-coated microparticles in the presence of Mg^{2+}. However, the presence of 1% pectinase led to an important increase of the drug release from the microparticles where the pectin was crosslinked with Ca^{2+} ions.

K-carrageenan/PVA hydrogel films, as wound dressings, were obtained by crosslinking with GA and used for the loading and release of gentamycin sulfate under physiological conditions (Bajpai et al. 2015). A diffusion-controlled release mechanism was noticed at 37 °C. In another study, k-carrageenan and hyaluronic acid (HA) were crosslinked with epichlorohydrin, and the obtained hydrogel films were loaded with L-carnosine, as a model drug (El-Aassar et al. 2015). The increase

of the HA content led to an increase of the porosity and thus to the increase of the water uptake. Moreover, the drug release was also affected by the HA content. Ciprofloxacin was loaded in β(1 → 3)(1 → 6)glucan/carrageenan (CG/car) hydrogels chemically crosslinked with GA (Nair et al. 2016). It was demonstrated that the presence of CBG enhanced the porosity, cell attachment, proliferation, drug loading, antibacterial activity, and wound healing ability of these hydrogels. The same drug was also encapsulated in hydrogels based on iota-carrageenan and gelatin, crosslinked also with GA (Padhi et al. 2016). The drug release was studied at pH 1.2 (gastric), 7.4 (physiological), and 9.4 (colonic). The faster drug release rate was noticed at pH 1.2. Moreover, the antimicrobial activity of these hydrogels was assessed. Pellets formulations were prepared based on k-carrageenan and starch hydrogels, crosslinked with glyoxal, and loaded with zaltoprofen (Sonawane and Patil 2018). The optimized formulation, with a 4:2 ratio of starch-carrageenan, displayed almost complete (99.15%) drug release after 12 h. In another study, k-carrageenan and β-cyclodextrin hydrogels were crosslinked with the same crosslinking agent and used for the loading of honey bee propolis extract (Sharaf and El-Naggar 2019). This hydrogel was further used for the impregnation of cationized cotton fabrics, and it appeared that this complex material has a high potential as wound healing system. Very recently, carrageenan, sodium alginate, and PEG, of different molecular weights, were used for the preparation of hydrogels by crosslinking with (3-aminopropyl)triethoxysilane for the encapsulation and release of lidocaine (Rasool et al. 2020). A cumulative release of around 85% was noticed after 6.5 h in PBS at 37 °C. Several theoretical mathematic models were applied in order to have a deeper insight of the drug release mechanism, and it was noticed that the drug release follows a quasi-Fickian diffusion model. At this point it is also of interest to mention the work of Akalin and Pulat (2020) in which k-carrageenan hydrogels were prepared by crosslinkinged with GA in order to be loaded with cooper and manganese micronutrients. Gel content increased from 72.3 to 93.4% as the amount of GA increased from 0.25 to 1.5 ml. As expected, both the swelling degree and drug release decreased with increasing the crosslinking agent amount.

Genipin was also used as crosslinking agent for the preparation of k-carrageenan/carboxymethyl cellulose (CMC) beads (Muhamad et al. 2011). Beta-carotene was loaded in these beads, and the swelling and the drug release were carried out under simulated gastrointestinal tract condition (pH 1.2 and 7.4). Moreover, the release rate was correlated with the crosslinking degree. A similar study was also performed by Hezaveh et al. (2012). In another study, these researchers have also loaded beta-carotene in k-carrageenan/hydroxyethyl cellulose and k-carrageenan/PVA hydrogels always crosslinked with genipin (Hezaveh and Muhamad 2013a, b). Using the same crosslinking agent, ranitidine HCl-loaded k-carrageenan/NaCMC hydrogels were prepared by another team of researchers (Selvakumaran and Muhamad 2015). These authors have studied the effect of the crosslinking degree, and it appeared that it had an influence on the gel strength, porosity, swelling, and drug release.

Isoniazid, as a model drug, was loaded in microspheres prepared from k-carrageenan and gelatin crosslinked with genipin (Devi and Maji 2010). A higher release rate was noticed at basic pH than at low pH values. Hydrogel microparticles

were also prepared from k-carrageenan using divinyl sulfone, as a crosslinking agent, in a sodium bis(2-ethylhexyl)sulfosuccinate reverse micellar system (Sagbas et al. 2012). Moreover, by loading Fe_3O_4 nanoparticles, hybrid hydrogel microparticles were obtained. The release studies were carried out in the presence of phenylephrine HCl, as a model drug, and a quaternization reaction of the chemical groups from the surface of these microparticles improved to a great extent the drug release in PBS.

Hydrogels based on hyaluronic acid (HA) were crosslinked with Gantrez S97 which is the acid form of a methyl vinyl ether and maleic anhydride copolymer (Larraneta et al. 2018). Methylene blue was used as a model hydrophobic principle and a sustained release was noticed during 2 days. In another study, ketoprofen-loaded HA was crosslinked in the presence of 2,2′-(ethylenedioxy)bis(ethylamine) (EDEA) and EDC (Csapo et al. 2018). Moreover, a cationic surfactant was used for the preparation of a hydrophobized HA by a partial neutralization with positively charged amines. The drug release kinetics was different for these two colloidal drug delivery systems. More recently, HA hydrogels, crosslinked with 8% adipic acid dihydrazide (ADH), were loaded with an anti-oxidative polyphenol (epigallocatechin gallate) used for the treatment of tendinopathy (Hsiao et al. 2019). A cumulative drug release of around 90% was noticed after 10 days and the therapeutic effect was assessed both in vitro and in vivo. Two drugs, such as curcumin and simvastatin, were loaded in nanogels based on thiolated HA and crosslinked with 1,4-bis(3-[2-pyridyldithio]propionamido)butane (Pedrosa et al. 2014). A mean diameter around 100 nm was observed by DLS, whereas the ZP value was −19.3 mV. Drug encapsulation, with the maximum efficiency of 52.1%, led to an increase of the mean diameter.

Agarose and Carbopol 974P were used for the preparation of resveratrol-loaded semi-IPN hydrogels (Tunesi et al. 2015). After 7 days, around 80% of drug was released indicating a controlled and sustained release.

Fucoidan-shelled CS beads were obtained using genipin as crosslinking agent and loaded with berberine for oral administration (Yu et al. 2015). The drug release rate was higher at pH 1.2 than at pH 7.4. Moreover, the drug-loaded beads inhibited the growth of both *S. aureus* and *E. coli*. In another study, fucoidan/CS-n-arginine nanogels were prepared by crosslinking with genipin and loaded with amoxicillin for preventing *Helicobacter* infections (Lin et al. 2017). The average sizes of these particles were in the range of 173 to 228 nm. The drug release was pH dependent, and it appeared that amoxicillin-loaded nanogels significantly inhibit *Helicobacter pylori* growth.

Chitin nanocrystals scaffolds were crosslinked with GA and encapsulated with curcumin-loaded Tween 20 micelles (Ou et al. 2018). Drug release rate was faster at pH 5.3 than at pH 7.4. Moreover, the cytotoxicity tests revealed the inhibitor effect of curcumin against the proliferation of MCF-7 cells.

2.2.1.2 Crosslinking by Grafting-Copolymerization

Synthesis of hydrogels based on polysaccharide modified with chemical, thermal, or photo-crosslinkable groups may provide an alternative method for hydrogel

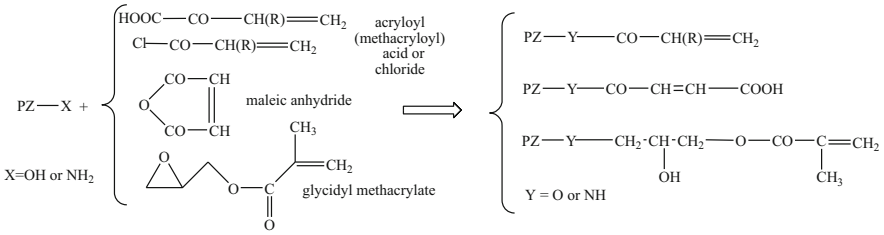

Fig. 2.2 Schematic representation of obtaining macromers by functionalizing the polysaccharide with polymerizable substituents

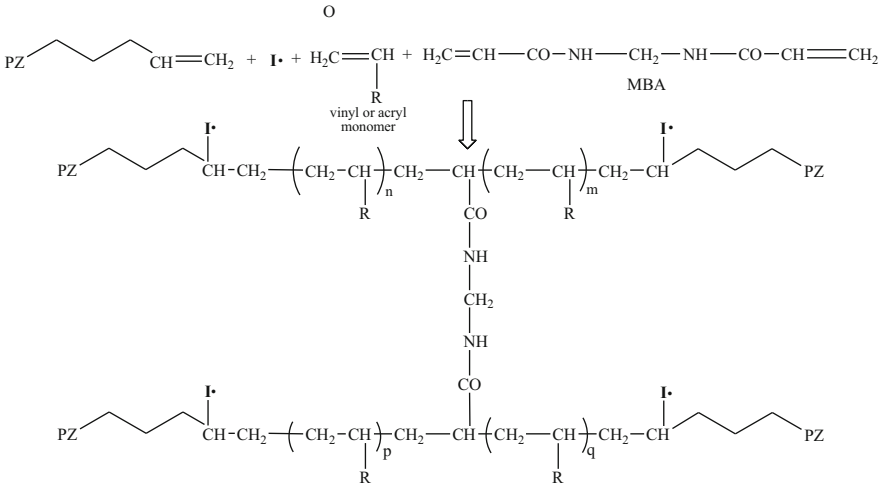

Fig. 2.3 Scheme of the polysaccharide macromer crosslinking (*PZ* polysaccharide chain)

formation; these hydrogels allow macromers containing maleic, itaconic, acrylic, or methacrylic groups as substituents, therefore being able to polymerize radicals and to gelate in situ. The introduction of the polymerizable groups to the polysaccharide base chain is done by analogous polymer transformation reactions to the OH or amine groups of the polysaccharide. Schematically, the functionalization of the polysaccharide, resulting in obtaining macromers, is presented in Fig. 2.2**.**

The copolymerization of the macromer with vinyl or acrylic monomers and with difunctional monomers that allow to obtain crosslinked structures (e.g., MBA), initiated chemically, thermally, or photochemically (with UV radiation or visible spectrum), subsequently leads to crosslinked structures which, given the general strong hydrophilic character of polysaccharides, have the character of a hydrogel (Fig. 2.3).

However, UV light initiation of polymerization has some drawbacks in tissue engineering applications such as DNA damage, immunosuppression, cell death, accelerating tissue aging, and cancer induction. Visible light initiation is preferred

because it could penetrate in the depth of tissues with lower energy than UV light and, consequently, it could reduce the DNA damages and increase cell viability chance. Therefore, visible light crosslinkable hydrogels are appropriate choices to develop injectable hydrogels.

Obtaining the crosslinked structures can also be done directly from the macromer by chemically or physically initiated copolymerization only with the difunctional monomer as crosslinker. Another way of obtaining hydrogels is the direct grafting/crosslinking of the polysaccharide, without being previously functionalized as macromer, in the presence or absence of vinyl monomers without requiring a difunctional monomer as crosslinker. The reaction is initiated chemically or physically by creating the polysaccharide macroradical, usually by extraction of a hydrogen atom from the -CH_2 group (C6 in the glucoside ring); in this case semi- or interpenetrated polymer networks (IPN) are obtained, the crosslinking occurring by recombining the macroradicals.

Among the first polysaccharides modified by the introduction of polymerizable groups as substituent on the base chain are gelatin and xanthan (which, however, have a nonmarine origin), transformed into maleate or acrylate macromers and subsequently polymerized/copolymerized with N-isopropyl acrylamide (NIPAM) and MBA in order to obtain thermosensitive hydrogels (Hamcerencu et al. 2007; Hamcerencu et al. 2012).

In a first stage, Wang et al. (2017) obtained an amide derivative of chitosan by the reaction of maleic anhydride to the NH_2 group of the polysaccharide. The synthesized macromer (CSMAH) is subsequently radically copolymerized with acrylic acid (AA) in the presence of ammonium persulfate (APS) as initiator. The obtained hydrogels are pH-sensitive, their degree of swelling in water increasing with the pH value and the amount of AA relative to CSMAH, ranging between 10 and 40 (w/w), depending on the value of the mentioned parameters. These are macrogel-type hydrogels, the size of the polymers decreasing with the increase of the AA weight in their composition, as a result of the increase of the crosslinking degree. The authors encapsulated amoxicillin and meloxicam in hydrogels and found that the drug release rates increase with pH increment and decrease with increasing the crosslinking degree. The kinetics of drug release are controlled to varying extents by a combination of diffusion and hydrogel relaxation.

pH-responsive hydrogel for controlled drug delivery was prepared from chitosan (CS), acrylic acid (AA), and (2-dimethylamino)ethyl methacrylate (DMAEMA) via in situ free radical polymerization (Che et al. 2016). In this case, it was not necessary to obtain a prior CS macromer, the crosslinking occurring by direct copolymerization of the two vinyl comonomers to the radicals created on the CS backbone under the action of the redox reaction initiation system (potassium persulfate and sodium thiosulfate). In fact, an IPN structure is obtained, the two networks being, on one hand, crosslinked CS and, on the other, a copolymer generated by the copolymerization of the two vinyl monomers. The obtained hydrogel has a pH-sensitive character, conferred by the coexistence in its structure of both basic groups (-NH_2 from CS) and acid (-COOH from AA). With these excellent features, this CS-based

pH-responsive hydrogel holds great promise to be utilized as vehicle for anticancer drug delivery.

Chitin is used mainly to obtain chitosan by deacetylation. Functionalization of chitin with photo-crosslinkable groups inserted in the base chain through the reaction with methacrylic acid is well known, and among the first published works was that of Tanodekaew et al. (2004). A UV photo-crosslinkable water-soluble chitin derivative was prepared recently by the action of methacrylic acid (MAA) on low molecular weight chitin under mild conditions in the presence of EDC and 4-dimethylaminopyridine as activators (Khor et al. 2011). Chitin methacrylate derivative (CM) was exposed to UV irradiation in the presence of a photo-initiator, resulting in a transparent hydrogel in about 10–15 min as indicated by a clear separation of a gel from the liquid. The CM-hydrogel is enzymatically degradable (lysozyme), after 24 h being obtained totally water-soluble compounds. In vitro tests for cytotoxicity against three cell lines, NCTC clone 929, IMR-90, and MG-63, indicated that the hydrogel was noncytotoxic, promoting the adhesion and proliferation of cells to its surface and suggesting the possibility of being used as support and for the inclusion of drugs.

Functionalization of alginic acid (AgA) or alginates with photo- or thermo-polymerizable groups is possible by using GMA which react by opening of the epoxy group with the -OH groups preferably from positions 2 and 3 of the glucoside ring. Thermally polymerizable AA-GMA macromers were thus synthesized under alkaline conditions (Wang et al. 2015). The AgA-GMA aqueous solution can be polymerized at physiological temperature (37 °C) in the presence of APS and N,N, N′,N′-tetramethyl ethylenediamine (TEMED) as initiator. After 5–20 min, in function of the concentration of initiator system, AgA-GMA hydrogels are obtained, exhibiting a porous structure with average pore sizes ranging from 50 to 200 μm, directly depending on the macromonomer concentration. The realization of hydrogels in these conditions makes it possible to obtain them by in situ polymerization after the intramuscular injection of the reaction system in experimental animals, finding at the same time only a mild inflammatory effect, which proves that the hydrogels are biocompatible. The biocompatibility of the hydrogels was also evaluated by human umbilical vein endothelial cell (HUVEC) encapsulation. It was noticed that both the cell viability and proliferation were unaffected by macromonomer concentrations, which suggests that AA-GMA has a potential application in the field of tissue engineering, as well as support for drugs immobilization.

Biocompatible hydrogel nanocomposites containing mesoporous silica were obtained starting from AA-GMA macromer, by copolymerization-crosslinking with 2-hydroxyethyl methacrylate (HEMA) and N,N-dimethylacrylamide (DMAAm) initiated by sodium persulfate. The hydrogels are biocompatible with human immortalized RWPE-1 prostatic epithelial cells (Hugo de Lima et al. 2018). The hydrogels showed higher drug (prednisolone) loading efficiency influenced by the increase in the amount of alginate and silica. The in vitro kinetic studies proved that delivery is produced with reduced burst effect (up to 90%) providing a steady release into the solution.

Agarose (AG) is a marine polysaccharide, generally used as a cell immobilization matrix, drug delivery vehicle, or dental impression material because of its gelling ability and biocompatibility. Few chemical modifications on AG have been done and thus a small number of AG derivatives were synthesized up to now. Acrylated AG-based hydrogels were reported by Pourjavadi et al. (2011), the potential application being that of systems for immobilization and sustained release of some fertilizers. Macromer (ACAG) was synthesized by esterification of AG with acryloyl chloride; three different substitution degrees (DS) macromers were obtained. Hydrogels were obtained by radicalic graft copolymerization of hydroxyethyl acrylate (HEA) and AA, initiated by APS. The properties of the obtained hydrogels depend on the DS value, which in fact determines the degree of crosslinking of the network. The morphology highlighted by MEB shows that these hydrogels have a highly porous structure, the pore size being smaller for the hydrogel obtained from the macromer with the highest DS. The results revealed that at high DS, water absorbency is decreased, an absolutely normal effect caused by increasing the crosslinking degree. Hydrosoluble compounds such as potassium nitrate can be loaded in these hydrogels; the loading efficiency and the release kinetics correlated well with the swelling results. The characteristics of the hydrogel and the ability to include and release water-soluble compounds in a sustained manner also recommend it as materials for obtaining sustained release polymer/drug systems.

Recently, Date et al. (2020a, b, c) report the synthesis of full-IPN hydrogels based on AG and poly(vinyl alcohol) (PVA) obtained by direct chemical crosslinking of the polymer mixture, without requiring the intermediate step of obtaining a macromer. Crosslinking is provided by MBA, being initiated by APS. Prepared hydrogels showed excellent swelling, high efficiency in loading inclusion complex of ibuprofen in β-cyclodextrin, and pH-dependent drug release. The biodegradation and cytotoxicity assay studies confirmed the degradable and nontoxic nature of hydrogels and prove that they are potential candidates for drug delivery.

The possibility of obtaining carbon dots-incorporated (CDs) hydrogel nanocomposites based on AG and PVA, as matrix for drug loading and release, was recently reported Date et al. 2020a, b, c. The presence of hydroxyl and carboxyl groups on surface of CDs makes it more hydrophilic, so that their incorporation in hydrogel matrices makes the obtained biocomposite to show excellent pH-responsive swelling and qualify them as excellent biomaterial for drug delivery applications. The biocomposite synthesis is achieved by crosslinking in solution the mixture of AG and PVA in which CDs were dispersed, using MBA as crosslinker and APS as initiator, in the presence or absence of norfloxacin (NFC) used as a water-soluble model drug.

The obtained biocomposite is pH-sensitive, and the degree of swelling in aqueous media increases with the amount of PVA in the network, respectively with the concentration of CDs. Hydrogels are biodegradable and noncytotoxic, capable of continuously releasing NFC for a long time (release efficiency of at least 30% after 12 h). It can be assumed these hydrogel nanocomposites, which act as double drug carrier system, are well suitable for controlled drug delivery.

In order to obtain superabsorbent materials with potential applications in agriculture, but also in the biomedical field, Chaudhary et al. (2020) synthesize a semi-IPN hydrogel, based on AG and gelatin. The semi-IPN structure is achieved by grafting/crosslinking of the mixture of two polymers (different ratios), microwave assisted, in the presence of methyl acrylate (MA) and (MBA). This hydrogel can be applied as a promising absorbent of water in agricultural applications, but its biodegradable and biocompatible character also recommends it for biomedical applications (drug release systems).

Hyaluronic acid (HA) is an attractive starting material for the preparation of hydrogels with desired morphology, bioactivity, and capacity to be associated with a variety of biological active compounds. The presence of the -OH and -COOH groups along the polysaccharide chain allows the realization of numerous functionalization reactions, including the introduction of unsaturated substituents. It can be crosslinked through a wide range of procedures, a review of which is published by Xu et al. (2012).

Macromers containing methacrylate groups were obtained by reacting HA with an excess of GMA or methacrylic anhydride (MeA) in aqueous media (Leach et al. 2003). Because the ester bond is susceptible to hydrolysis while the ether bond is not, the reaction conditions in this phase determine the degradability of the hydrogels obtained from these precursors. GMA-HA hydrogels were obtained by exposing the macromer for 1 min to UV light in the presence of the photo-initiator Irgacure 2959 and N-vinyl pyrrolidinone as comonomer. The properties of the network can be shaped by modification of the HA molecular weight, degree of methacrylation, and the concentration of the macromer.

Chemically modified HA with polymerizable groups provides a more efficient means to produce micro-/nanoparticles. For example, radical polymerization of HA-GMA in an inverse emulsion of water in hexanes containing Span 80 and Tween resulted in nanogels of ~30 nm in size with an estimated swelling ratio of ~10 g/g (Prata et al. 2010). Microgels were obtained from precursor solution of methacrylated HA and a photo-initiator in water which was deposited onto PDMS pattern; the photo-crosslinking occurred by the exposure to the UV light. When cells are included in the precursor liquid, cell-laden microgels are prepared (Yeh et al. 2006).

Fucoidan (Fu) photo-crosslinked structures for biomedical applications were obtained by Reys et al. (2016) starting from a methacrylated fucoidan (MFu) macromer. MFu was synthesized by reacting Fu with methacrylic anhydride, in aqueous solution at pH 8, for 6 h at 50 °C. To obtain MFu particles, a technology based on superhydrophobic surfaces was used. Drops of 1 to 5 μl of MFu and photo-initiator solution were dispersed onto the superhydrophobic surface, forming spherical particles with different sizes. The surfaces drop-loaded were exposed to visible light for 15 min to promote photo-crosslinking. The diameter of the obtained particles, which present a spherical shape, varies between 2 and 20 mm (wet state), respectively 0.9 and 1.3 mm after lyophilization, the smallest average diameter being obtained when using higher concentrations of MFu.

The cytocompatibility of the developed structures was evaluated by culturing L929 cells in direct contact with the MFu and photo-crosslinked particles and evaluating cell metabolic activity (MTS assay), proliferation (DNA quantification), adhesion, and morphology (CLSM). The particles proved to be not cytotoxic and could be good candidates as cell culture matrices or drug delivery vehicles for biomedical applications.

Injectable hydrogels are promising materials for achieving hemostasis in case of internal injuries and bleeding, as these biomaterials can be introduced into a wound site using minimally invasive approaches. An ideal injectable hydrogel precursor should solidify after injection in the wound area and promote the natural clotting cascade.

Carrageenan is a polysaccharide that, by introducing unsaturated groups into the base chain, can generate injectable macromers, which can polymerize once introduced into the body. Injectable hydrogel based on visible-light crosslinked methacrylate-kappa-carrageenan (KaMA) was recently reported by Tavakoli et al. (2019). The macromer KaMA with two different degrees of methacrylation (6 and 12%) was primarily synthesized by esterification of polysaccharide with methacrylic anhydride, in solution, at pH 8. To synthesize visible-light crosslinked KaMA hydrogel, different amounts of KaMA were dissolved in water; photo-initiator (eosin Y), the co-initiator (triethanol amine), and comonomer N-vinylcaprolactam were added. The final solution was injected on a glass coverslip and exposed under white LED light (100 Mw. cm^{-2}) for 180 s. The chemical crosslinked gel thus obtained was gently sprayed with a solution of KCl physical crosslinking. From a morphological point of view, the hydrogels are macroporous, the pore size decreasing with the increase of the methacrylation degree. The cytocompatibility of these hydrogels was investigated via in vitro viability, metabolic activity, and proliferation of cells, via surface seeding. For investigating degradation behavior of KaMA in vitro, the samples were incubated in PBS at 37 °C for different time periods (3, 7, and 14 days). Evidently, the degradation rate of all samples enhanced with increasing incubation time. Higher methacrylation degree led to denser and packed hydrogel networks and resulted in reduced hydrogel degradation in PBS. The results proved the potential of visible-light crosslinked KaMA hydrogels to be used in tissue engineering applications without any harmful side effects and suggest the possibility of using them as carriers of drugs in the treatment of skin conditions (burns, incisions, excisions).

pH-sensitive and superabsorbent k-carrageenan (kC) hydrogels were synthesized by grafting/crosslinking of polysaccharide with different vinylic monomers (or mixtures) initiated by classical initiators or redox systems. Sadeghi and Soleimani obtained pH-responsive and low salt-sensitive superabsorbent hydrogels by crosslinking/graft copolymerization of acrylamide (AAm), AA, or MAA onto kC backbone in solution (water), in the presence of crosslinker (MBA) by using APS as initiator (Sadeghi and Soleimani 2011). In the case of AA and MAA, some carboxylic groups can be converted to carboxylate anions which contribute to the amplification of the superabsorbent character of the hydrogel. In the case of AAm, the network may be hydrolyzed under alkaline conditions. Hydrogels have a high

swelling capacity in aqueous solutions of LiCl, NaCl, and KCl, due to anti-salt characteristics of the sulfate groups from kC part of the network. The pH-sensitive character and the swelling-deswelling behavior of the hydrogels make them suitable biomaterials to design new systems for controlled drug delivery. The same group obtained superabsorbent hydrogels by grafting/crosslinking of AAm and itaconic acid (IA) onto kC backbone, followed by alkaline hydrolysis (Sadeghi et al. 2012). The swelling capacity in water was proved to be dependent on the crosslinker (MBA) and of the monomer mixture concentration. Higher swelling capacities were obtained from employing higher initial ratios of IA/AAm. Although the authors did not study this aspect, the high degree of swelling in the water and the pH-sensitive character recommend the hydrogels obtained for the realization of sustained release systems of water-soluble drugs.

A pH-responsive hydrogel was obtained, too, by grafting/crosslinking of kC with AA, in the presence of MBA as crosslinker and using APS as initiator (Hosseinzadeh 2010). Diclofenac sodium salt was loaded by diffusion in these hydrogels, the loading efficiency depending on drug concentration, loading time, and crosslinking degree of the network. The release of diclofenac from the hydrogels decreases as the pH of the dissolution medium was lower. At low pH values, electrostatic repulsion between the carboxylic acid groups of backbone is low, thus decreases gel swelling and minimizes release of drug by diffusion. In alkaline media the electrostatic repulsion between carboxylate groups increases, thus increasing the gel swelling degree, and so the release of drug increased.

Synthesis of k-carrageenan-based superabsorbent hydrogels with improved properties was reported by Salimi et al. (2010). They are obtained by homogenous solution polymerization process, starting from kC, AA, sodium acrylate (NaA), 2-hydroxyethyl acrylate (HEA), and MBA, by using APS as initiator. The hydrogels showed interesting properties when the swelling medium was alternatively changed between solutions with different salt concentrations and between various water-methanol mixtures with altered compositions. This smart behavior may be exploited for obtaining biomedical applications, such as systems for drug delivery.

A biopolymer-based superabsorbent hydrogel composite based on k-carrageenan (kC) has been prepared via graft copolymerization of AA in the presence of bentonite powder using MBA as a crosslinking agent and APS as an initiator (Hosseinzadeh et al. 2011). The swelling properties of biocomposites proved to be a function of the monomer, initiator, crosslinker, and bentonite concentration. Generally, swelling ability of these "anionic" hydrogels in salt solutions is appreciably decreased compared to the swelling values in distilled water. The authors studied the swelling of kC/bentonite-based biocomposites in saline solutions of NaCl, $CaCl_2$, and $AlCl_3$. The decrease of water absorbency in multivalent cationic solutions could be related to the complexing ability of the carboxylate groups to form polymeric matrix with the formation of intramolecular and intermolecular complexes which resulted in an increase of the crosslinking degree. Another superabsorbent hydrogel biocomposites were obtained by using montmorillonite (MMT) particles as mineral filler. For example, Sadeghi et al. (2012) reported a hydrogel composite based on kC grafted-crosslinked with AAm, IA, and MBA, in the

presence of montmorillonite. The biocomposite possesses high biocompatibility and biodegradability (due to the kC part) and higher swollen gel strength (due to the inorganic parts).

Highly swollen *iota*-carrageenan (iC) and MMT-based hydrogels were prepared by free radical crosslinking copolymerization of the polysaccharide with AAm and an anionic comonomer such as sodium methacrylate (SMA); poly(ethylene glycol) diacrylate (PEGDA) was used as a crosslinker and APS as initiator. The hydrogels, of semi-IPN type, and the hybrid composite systems thus obtained, have the ability to absorb water in high amounts and could serve as a potential device for water or dye sorbents with potential applications in agriculture, environment, separation processes, and water purification. Evidently, they can include hydrosoluble drugs and could also be used as supports for the inclusion/release of drugs (Karadağ et al. 2014).

Recently, agar/kC and agar/kC/MMT hydrogels were prepared by the free radical crosslinking reaction of AG and kC in the presence of triethylene glycol divinyl ether (TEGDE), APS, and (TEMED) used as initiator-accelerator system (Gürkan Polat et al. 2020). Hydrogels with various swelling degrees were obtained by adjusting the parameters of reaction (free radical initiator and crosslinking agent concentrations, reaction temperature, kC/AG ratio, and MMT concentration). They could be potentially used as eco-friendly and nontoxic materials for biomedical applications (tissue engineering, drug release).

2.2.2 Ionic Crosslinking

Marine polysaccharides contain in their native structure ionizable groups, or they can be created at a later stage of processing, as is the case of chitosan obtained by deacetylation of chitin. The presence of these ionic or ionizable groups makes it possible to crosslink by interposing di- and polyfunctional ions between the linear polymer chains. The great advantage of the process is that it does not use toxic crosslinking agents; the disadvantage is the low chemical and mechanical stability of the obtained hydrogels. By ionic crosslinking various drug delivery systems can be obtained in the form of micro-/nanoparticles, capsules, and films for controlled and sustained release of active therapeutic principles.

The literature abounds with examples of hydrogels obtained by ionic crosslinking, sometimes combined with other types of crosslinking. The majority of marine polysaccharides are anionic, so the crosslinking agents used are di- and polyvalent cations: Ca^{+2}, Mg^{+2}, Fe^{2+}, Fe^{3+}, Zn^{2+}, and Cu^{2+}. An example of a cationic polysaccharide is chitosan (CS), so its crosslinking is done with di- and polyanions (sulfate anion, tripolyphosphate). A selection of works published in the last 10 years, highlighting the type of ionic crosslinker, the formulation of the hydrogel, and the encapsulated drug, is presented in Table 2.1.

Numerous hydrogels have been reported by crosslinking alginate with $CaCl_2$ (Zhang et al. 2019a, b; Patel et al. 2016; Abdellatif et al. 2020; Uyen et al. 2020), mixtures of it with CS (Feng et al. 2014), chondroitin sulfate (Fajardo et al. 2012),

Table 2.1 Drug-loaded ionic crosslinked hydrogels

System	Crosslinking agent	Loaded drug	Type of formulation	References
SA	$CaCl_2$	– Insulin – Doxorubicin – Ibuprofen – Ketoprofen, quercetin – Ceftriaxone sodium – Metformin – Indometacin – Metformin HCl – Hydrocortisone – Acetaminophen – Ibuprofen – Retinoic acid – Dexamethasone	– Microparticles – Nanogel – Beads – Hydrogel – Microparticles/ beads – Microparticles – Beads – Microparticles – Microparticles – Hydrogel – Aerogel beads – Microparticles – Hydrogel	Ahmed and El-Say (2014) Xue et al. (2015) Dalaty et al. (2016) Gonçalves et al. (2016) Patel et al. (2016) Nayak et al. (2016) Cerciello et al. (2017) Samak et al. (2017) Yin et al. (2018) Szekalska et al. (2018) Veres et al. (2018) Wang et al. (2018) Zhang et al. (2019a, b)
	$Zn(CH_3COO)_2$ $FeCl_3$ $CaCO_3$	– Curcumin – Cetuximab/ octreotide – Prednisolone	– Microparticles – Beads – Beads	Uyen et al. (2020) Abdellatif et al. (2020) Russo et al. (2020)
Sodium hyaluronate	$CaCl_2$	– Tocopherol	– Nanoemulsion	Kong and Park (2011)
Kappa-carrageenan	NaCl, KCl, $CaCl_2$, $MgCl_2$, KCl, KSCN KCl, CaCl2	– Ibuprofen – Curcumin – Tetracycline	– Aerogels – Hydrogel film – Aerogels	Obaidat et al. (2018) Kadota et al. (2020) Agostinho et al. (2020)
CS	TPP	– Clobazam – Docetaxel – Acetaminophen	– Microspheres – Nanoparticles – Tablets with NPs	Kumar et al. (2012) Mahmood et al. (2019) Pinto et al. (2018), Pan et al. (2020)
Chitin	TPP	– Paclitaxel – Ethionamide, methacycline, rifampicin	– Nanoparticles – Nanoparticles	Smitha et al. (2013) Gayathri et al. (2017)

(continued)

Table 2.1 (continued)

System	Crosslinking agent	Loaded drug	Type of formulation	References
	$CaCl_2$	– Methotrexate	– Nanogels	Panonnummal and Sabitha (2018)
SA/CS	TPP + $CaCl_2$ TPP + $CaCl_2$ $CaCl_2$ TPP	– Doxorubicin – Rabeprazole – L-ascorbic acid – Vancomycin HCl	– Beads – Nanoparticles – Membranes – Microparticles beads	Feng et al. (2014) Ahmed and El-Say (2014) Gierszewska et al. (2018) Unagolla and Jayasuriya (2018)
SA/CS micelles	$CaCl_2$	– Emodin	– Microparticles	Conga et al. (2018)
SA/locust bean gum	$AlCl_3$	– Capecitabine	– IPN microbeads	Upadhyay et al. (2018)
SA/chondroitin sulfate	$CaCl_2$	– Indomethacin	– Hydrogel	Fajardo et al. (2012)
SA/sterculia gum	$BaCl_2$	– Pantoprazole	– Microparticles beads	Singh and Chauhan (2011)
SA/starch	$CaCl_2$	– Diclofenac sodium	– Microparticles	Khlibsuwan et al. (2018)
SA/ carboxymethyl cashew gum	$ZnSO_4$	– Isoxsuprine HCl	– Hydrogels microparticles	Das et al. (2014)
SA/CMC	$FeCl_3$	– Metformin	– Hydrogel beads	Swamy and Yun (2015)
SA/guar gum	$BaCl_2$	– Ibuprofen	– Hydrogel beads	Seeli et al. (2016)
SA/gelatin/ hydroxyapatite	$CaCO_3$	– Tetracycline HCl	– Hydrogels	Yan et al. (2016)
SA/ montmorillonite	$CaCl_2$	– Acetaminophen	– Hydrogels	Eral et al. (2014)
SA/CS/k-carrageenan	$CaCl_2$, KCl	– 5-Fluorouracil	– Microbeads	Sun et al. (2019)
SA/κ-carrageenan	$CaCl_2$	– Insulin	– Hydrogel beads	Lim et al. (2017)
CS/k-carrageenan	TPP	– Hydrophilic drugs	– Nanoparticles	Rodrigues et al. (2012)
CS/fucoidan	TPP	– Gentamicin – Ciprofloxacin	– Nanoparticles – Nanoparticles	Huang et al. (2016) Elbi et al. (2017)
HA/CS	TPP	– Curcumin, resveratrol	– Nanoparticles	Hussain et al. (2020)
SA/carbon dots	TPP	– Tetracycline HCl	– Microparticles	Gogoi and Chowdhury (2014)

starch (Khlibsuwan et al. 2018), locust beam gum (Upadhyay et al. 2018), k-carrageenan (Lim et al. 2017; Sun et al. 2019), or powdered inorganic substances such as hydroxyapatite (Yan et al. 2016) and montmorillonite (Eral et al. 2014). Obviously, such combinations were made in order to regulate some properties of the hydrogel, such as hydrophilicity, the ability to include and release the drug. The use of metal cations such as Zn^{2+}, Cu^{2+}, and Fe^{2+}/Fe^{3+} can result in obtaining more stable hydrogels, as a consequence of the additional coordination bonds formed by these cations. Using metal cations can also crosslink fucoidan, chondroitin sulfate, HA (Kong and Park 2011), or alginate (Ghosal et al. 2020), but the lower stability of the gels they form makes these polysaccharides to be used mainly in combination with the aforementioned or in the form of derivatives. CS/fucoidan-based NPs, for example, loaded with gentamicin, have been evaluated physicochemically and biologically for pulmonary drug release, with increased antibiotic efficacy and elimination of side effects (Huang et al. 2016).

Chitosan is practically the only cationic polysaccharide, although sporadically chitin also appears in some works. The crosslinking should be done with the SO_4^{2-} anion or with phosphate polyanions (Kumar et al. 2012, Pinto et al. 2018, Szymańska et al. 2020); thus the obtained drug-loaded systems (films, micro-/nanoparticles) have the advantage of bioactivity (antimicrobial) of the polysaccharide. Anion-crosslinked chitin-based hydrogels are less present in the literature (Smitha et al. 2013; Gayathri et al. 2017).

The drug-loaded ionically crosslinked hydrogels based on marine polysaccharides have numerous biomedical applications, such as drug delivery systems for oral (Samak et al. 2017), vaginal (Pavelkova et al. 2017), subcutaneous (Zhang et al. 2019a, b), and parenteral injection route administration (Uyen et al. 2020); systems for targeting colorectal cancer (Sun et al. 2019), for pulmonary sustained and controlled delivery (Huang et al. 2016), and for the treatment of proliferative vitreoretinopathy; gastro-retentive floating drug delivery system (Nayak et al. 2010); systems with systemic antidiabetic action (Lim et al. 2017); system with antibacterial properties (Gayathri et al. 2017); system for psoriasis treatment (Panonnummal and Sabitha 2018); and system for the treatment of intracellular and biofilm infections of *Salmonella* (Elbi et al. 2017).

2.3 Physically Crosslinked Hydrogels

Another way to obtain hydrogels, including those based on marine polysaccharides, is the physical crosslinking, respectively obtaining non-covalent, nonionic, and non-coordinative interactions between macromolecules. Such hydrogels are of great interest due to the absence of chemical crosslinking agents and reagents that are usually toxic and which must be detached or isolated frequently from prepared gels before application. Presence even in small quantities of these compounds can affect the nature of the substances when entrapped (drugs, proteins, enzymes, cells). Because the physical crosslinked hydrogels depend on temperature, pH, or ionic strength changes, they are considered "smart materials." However, the great

disadvantages for this type of hydrogels are their instability (uncontrolled dissolution may occur), low mechanical resistance, difficult control of pore size, and network faults due to the free chain ends or chain loops (Hamedi et al. 2018). The formation of physical hydrogels is based on the reversible interactions that can occur between polymer chains, mainly hydrophobic interactions, hydrogen bonds, presence of crystallites for some polymers, and interactions dependent of parameters such as pH, concentration, temperature, etc. Obviously, the properties of these hydrogels may be adjusted according to the intensity and number of these interactions.

Polymers containing hydrophilic and hydrophobic domains, so having an amphiphilic character, can crosslink in aqueous media by reverse thermal gelation, the so-called sol-gel chemistry. The amphiphilic polymers are generally water soluble at low temperature, but, when temperature increases, gelation occurs. The temperature at which gelation occurs depends on the polymer solution concentration, length of the hydrophobic block, and, of course, the polymer chemical structure (Hoare and Kohane 2008). In the case of hydrophilic polymers, specific to polysaccharides, hydrophobic domains can be created by introducing long alkyl chains as substitutes to the backbone. For example, by *N*-acylation of CS with fatty acyl chlorides, hydrophobic interactions appear, enhancing the stability of substituted polysaccharide via hydrophobic self-assembly (LeTien et al. 2003). By adjusting the density and length of alkyl substituents, the degree of swelling and diffusion of the drug can be controlled. A potential application is the local and controlled release of growth factors in tissue repair. It should be noted that CS is able to form a gel by itself, without the need of any additive. This effect is possible by neutralizing the amino groups, determining the drastic reduction of the rejections between the macromolecules, increasing the approach of the chains, resulting in the formation of the hydrogel via hydrogen bonds, hydrophobic interactions, and CS crystallites (Croisier and Jérôme 2013).

Agarose can easily form a hydrogel without the presences of toxic crosslinking agents and catalysts. At high temperature, agarose chains in solution have a statistically coiled conformation (random coil), but by heating they form single or double helical structures which subsequently aggregate, forming a bundle and then a gel called "thermoreversible hydrogel." Depending on the concentration, the helical chains can aggregate and form clusters, which, at high concentration, become interconnected and generate a hydrogel-type network. The presence of hydroxyl (−OH) groups on the agarose structure facilitates self-gelling via hydrogen bonding (Le Goff et al. 2015, Zarrintaj et al. 2018, Manouchehri et al. 2018). The typical physically crosslinked agarose hydrogels were investigated for biomedical applications, such as tissue engineering (Singh et al. 2016) and drug release (Date et al. 2020a, b, c).

kC has also thermoreversible gelling capacity, the gelling process being due to the conformational change from coil to helix conformation, transition succeeded by helix aggregation (Chronakis et al. 2000). The realization of these conformational changes is done by ionic interactions and hydrogen bonds as result of the cooling of the polymer solution in the presence of some salts. This property of the polysaccharide to physically crosslink, as well as its biocompatible character, has been

exploited in biomedical applications such as tissue engineering and drug delivery systems. However, for achieving viable systems with the possibility of application in these fields, it is necessary to increase the thermal stability, mechanical properties, and efficiency of the drug release process. One option is the realization of nanocomposites with kC matrix, nature of filling material being diverse (Salgueiro et al. 2013).

kC/halloysite nanotube (HNT) nanocomposite hydrogels were synthesized via physical crosslinking for the gastrointestinal tract release. The incorporation of HNT enhanced the thermal stability, swelling degree, as well as drug loading and release behavior, compared to the pure kC hydrogel. In vitro cytotoxicity test revealed that the obtained hydrogels are biocompatible, so they may have great potential for applications, for example, in oral drug delivery systems (Sharifzadeh et al. 2016).

Among synthetic polymers, PVA has been widely used in biomedical applications because it has excellent chemical and physical properties, as well as biodegradability. It can form physical crosslinked hydrogels by freeze-thaw processing. During the freezing stage, a phase separation occurs: one of the phases is poorer in polymers, when ice crystals are produced, while in the polymer-richer phase, the formation of hydrogen bonding and PVA crystallites takes place. The thawing stage facilitates the interactions and formation of crystalline regions between the remaining polymers, resulting in formation of hydrogel networks. Many polysaccharides have been added to the PVA aqueous solution to form hydrogels (cryogels) which combine the properties of both materials.

Sodium alginate (SA) has also been introduced into PVA solution to fabricate composite cryogels using freeze-thawing method. The hydrogels were loaded with nitrofurazone, a topical anti-infective drug, and could have applications as wound dressing systems (Kim et al. 2008).

Starting from mixtures in PVA and carrageenan solution, subjected to five consecutive frosting cycles (24 h, −20 °C)/thaw (room temperature, 6 h), there were obtained composite hydrogels possessing a larger pore size and highly interconnected porous structures while maintaining their original shape. They can significantly promote cell attachment and proliferation (ATDC5 cells cultured). It is evident that these composite hydrogels could serve as an excellent scaffold for tissue engineering, as well as for drug loading and release (Zhang et al. 2015a, b). New PVA/*iota*-carrageenan hydrogels obtained by consecutive freeze-thaw cycles were reported recently by Croitoru et al. (2020). The formation of the hydrogel polymer matrix is due to interactions through hydrogen bonding and forming of mixed interpolymer crystalline domains. These hydrogels have the ability to be loaded with antibiotics (amoxicillin, tetracycline hydrochloride, and gentamicin sulfate), and their release being strongly dependent on the drug chemistry, mesh size of the hydrogels, swelling mechanism, and pH of the release medium. Moreover, the drugs release is decreasing with the increase in carrageenan content. Similarly, agar and PVA-based physical hydrogels were obtained after a single freeze-thaw cycle applied to the solution of the two polymers. The hydrogel shows excellent self-healing properties without adding other healing agent, enhanced thermal and mechanical properties (Shao et al. 2019).

Hyaluronic acid (HA) can form cryogels through repeated freeze-thaw cycles, without being associated with PVA. The main procedures of their preparation include acidifying HA solution and then freeze-thaw of the acid HA solution at proper subzero temperature by once or repeating freeze-thaw (Luan et al. 2012).

The physical-chemical and morphological characterization of hydrogels demonstrates that they belong to the category of physically crosslinked gels whose three-dimensional structure is stabilized mainly by multiple interchain hydrogen bonds in the junction zones of the polymeric network.

2.4 Double Crosslinking

Double crosslinking has become a process for obtaining hydrogels due to the need to improve some of their properties, especially mechanical ones. The process involves the combination, usually of covalent bonds and ionic bonds, with complexing or physical crosslinking. Covalent crosslinking combined with ionic one is used for polymers with ionizable groups, or for mixtures of polymers of which at least one has an ionic character. It is well known that covalent crosslinking with difunctional agents such as epichlorohydrin, glutaraldehyde, etc. imprints cytotoxicity and reduces biocompatibility on the hydrogels thus obtained, so it is recommended to avoid them. On the other hand, covalently crosslinking hydrogels show superior mechanical properties compared to the ionic ones, but both their swelling properties and the loading/release capacities are inferior. Ionic crosslinking itself does not involve the use of toxic crosslinkers, but it is difficult to obtain uniform hydrogels, stable in time and with good mechanical properties. As a result, double ionic and covalent crosslinking was required to obtain stable but also biocompatible, respectively, nontoxic hydrogels, usable for biomedical purposes.

In situ forming chitosan (CS) hydrogels have been prepared via double ionic crosslinking (with glycerolphosphate complex as crosslinker) and covalent (genipin as crosslinker) (Moura et al. 2011). Different amounts of genipin were used to form the crosslinking structure and performance. The hydrogels are highly porous with interconnecting pores, degradable in the presence of lysozyme and nontoxic. By injecting the in vivo formulation, the hydrogel forms rapidly and remains stable at the injection site for at least 1 week.

Some papers report the execution of hydrogels (films, micro-/nanoparticles) by double crosslinking, after the previous chemical modification of chitosan by new functional groups or by its grafting.

Chitosan NPs suitable for drug delivery applications were recently reported by Dmour and Taha (2017). The polysaccharide was first grafted to phthalic, succinic, glutaric, and phenylsuccinic acids, which allowed the insertion of -COOH groups to polymer backbone. Subsequently, the chitosan carboxylated derivative was first crosslinked by ionotropic gelation using tripolyphosphate (TPP) anion and, further, covalently, in the presence of EDC, which activates the condensation reaction of the amino groups of CS with the newly inserted carboxylic ones. The cytotoxicity of Dox was enhanced upon loading in ionotropic or covalent NPs compared to free

drug. In another study, the carboxylated CS was firstly ionically crosslinked with polyphosphoric acid (PPA), hexametaphosphate (HMP), or TPP and further, covalently, in the presence of EDC, thus creating amide bonds between backbones of polysaccharide (Saeed et al. 2020). The mean diameters of the spherical particles range between 120 and 320 nm. The characterization methods applied suggest that HMP resides within NPs cores, while TPP and PPA act mainly as NPs surface crosslinkers. The NPs showed high loading capacity of Dox and higher cytotoxic properties against MCF-7 cancer cells as compared to free drug. Further studies are necessary on biodegradability and elimination before moving on to clinical trials.

A hydrogel film was prepared from carboxymethyl chitosan (CMCS) and carboxymethyl cellulose (CMC) by ionical and covalent crosslinking with $CaSO_4$ and genipin, respectively. (Bao et al. 2014). The tests indicated that the prepared hydrogels possess the swelling-deswelling properties in a wide pH range. The gels are biocompatible and nontoxic, as suggested by the cells comparatively cultured on the crosslinked hydrogels and the negative and positive controls. Considering the useful properties, this kind of hydrogel films have potential application in drug delivery vehicles and skin tissue engineering. However, the use of unmodified chitosan for obtaining hydrogels is limited by its insolubility in water but only in acidic environment; obviously, this requires an advanced purification of the obtained products. Therefore, a possibility to increase the solubility in water is determined by the chemical modification of the polysaccharide, one possibility in this sense being its grafting. Chitosan-graft-poly(ethylene glycol) methacrylate with high solubility in water was obtained via Michael addition, in order to prepare potentially nontoxic micro-/nanoparticles (Savin et al. 2019). The technique selected for the preparation of the NPs was a double crosslinking (ionic and covalent) process in reverse emulsion which provides the mechanical stability of the polymeric nanocarrier. NPs' potential as drug delivery system for the treatment of posterior segment of the eyeball was analyzed by loading bevacizumab (BEV), a full-length monoclonal antibody. The NPs proved to be noncytotoxic and biocompatible, and released BEV in a controlled manner for several days (which may extend up to 14–30 days), so they could be a potential carrier for controlled release of BEV as an ophthalmic drug delivery system to the retina. Another CS derivative synthesized by polysaccharide functionalization with folic acid was utilized to obtain NPs with potential applications in cancer therapy. The NPs, loaded with an antitumoral drug, are able to target the tumor cells that present in membrane a receptor capable to recognize the folic acid (Alupei et al. 2017).

Some papers published in recent years report the production of double crosslinked hydrogels, starting from mixtures of CS with other polymers (proteins, polysaccharides), most often natural or even synthetic. One paper reports the preparation of micro- and nanoparticles of interpenetrated/interconnected network type, having a hydrogel character, based on CS and gelatin (G) suitable for application in ocular drug administration, by a two-step crosslinking process performed in reverse emulsion separation system. To reduce the amount of covalent crosslinker, more often toxic, without substantially changing the mechanical stability and at the same time maintaining a high swelling capacity in aqueous media that will further

induce the possibility of high hydrosoluble drug loading, the research group of Prof. Popa proposed an original method for the preparation of CS-based double crosslinked hydrogels (Peptu et al. 2010). In a first stage, the ionic crosslinking is achieved by using sodium sulfate; in a second one, the covalent crosslinking is achieved by using a low amount of GA in order to stabilize the shape, dimensions, and the stability of the obtained particles. The microparticles manifested pH-sensitive interactions in aqueous environments, high swelling capacity, high capacity to load, and release hydrophilic drugs (adrenaline) which can be controlled by modulating the initial ratio of the two polymers. The in vivo biocompatibility tests showed that microparticles promoted fibrillogenesis. Clinical tests showed that the microparticles were easy to administer and well tolerated with no visible side effects. The same group obtained new G- and CS-based hydrogels, by a double crosslinking method which is partially different as compared to the previous one: (i) the first stage of the synthesis was the covalent crosslinking, followed by the ionic one; (ii) the ionic crosslinker used was the TPP (Jătariu et al. 2011). The authors use hydrogel as support for inclusion, respectively the release of caffeine, chosen as a model drug. The higher amount of CS in the initial mixture increases the maximum swelling degree and the maximum amount of loaded/released caffeine. Starting from CS and G, another hydrogel was obtained, using sulfate anions as ionic crosslinker. The covalent crosslinking consists in the reaction between carbonyl groups of GA with free amine groups from the two polymers (the amount of GA is reduced and assures the crosslinking of 20% from amino groups); the ionic crosslinking is based on sulfate anions interaction with protonated amine groups of the polymers solubilized in acetic acid. Rheological tests were performed to study the mechanical stability of the hydrogels, and, generally, the results show elastic and stable crosslinking. The loading/release properties of caffeine from the hydrogels could be easily controlled by adjusting the studied parameters: ratio between polymers (G/CS), amount of ionic crosslinker, and crosslinking time (Jătariu et al. 2013).

The previously obtained results were extremely useful for the subsequent preparation of NPs as potential drug carriers. CS- and G-based NPs were prepared by double crosslinking in the droplets formed by reverse W/O emulsion method. The ionic crosslinking using SO_4^{2-} ions polymers gelation was followed by a covalent crosslinking with GA (Jătariu et al. 2012). The particles have a high capacity to swell in water and to include water-soluble drugs, for example, chloramphenicol (CFN). In vivo biodistribution studies proved the fluorescein-marked NPs capacity to penetrate various organs when they are administered via intraperitoneal and intravenous routes to Wistar male rats. CS-/G-based submicronic capsules were prepared by double crosslinking (ionic and covalent) in an O/W/O double emulsion by the same group. The variation of polymer/ionic crosslinker molar ratio had an influence on particle size, morphology, swelling degree, as well as their capacity to incorporate and release active principles (Moise et al. 2012). New active principles with potential antitumoral activity, derived from L-phenylalanine, were synthesized and encapsulated in these NPs. Antitumoral potential of the drugs, in free form or when incorporated into polymer NPs, against the development of Guerin's carcinoma has been proven to enhance their biological activity.

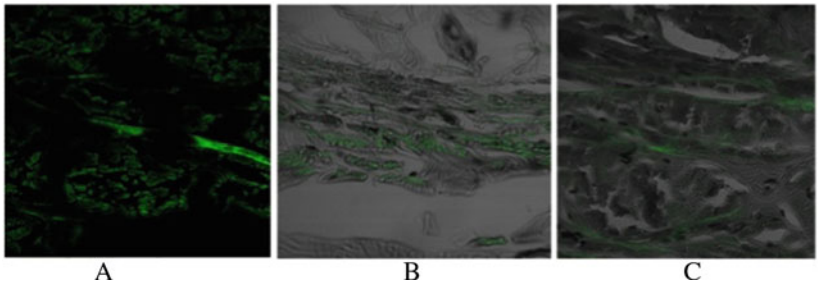

Fig. 2.4 Biodistribution of fluorescently tagged CS- and G-based covalent and ionically crosslinked NPs for the eyeball. (**a**) Retina, (**b**) sclera and extrinsic muscles, (**c**) optic nerve

Another study aimed to develop and characterize a new ophthalmic formulation of cefuroxime, using CS- and G-based NPs synthesized by ionic and covalent double crosslinking, by a double emulsion technique. The particles considered optimal have been analyzed, based on the enzymatic degradation properties along with in vivo ocular investigations (ocular biodistribution, in vivo drug release) (Andrei et al. 2015). The biodistribution test of the fluorescent-labeled NPs intravitreally injected demonstrated their preferential location in the retina (Fig. 2.4) and to a lesser extent in the sclera and extrinsic muscles of the eyeball (**b**) and in the optic nerve (**c**).

Small NP's diameter, biodegradability and non-toxicity, ability to locate especially in the retina, in vivo testing in guinea pigs of NPs loaded with cefuroxime that demonstrated their ability to deliver the drug in a sustained manner for at least 100 h at a therapeutic level, all these advantages recommend this system as an alternative to the classic treatment for diseases of the posterior side of the eyeball (retinitis, uveitis, macular degeneration).

An in situ forming and thermosensitive CS/G hydrogel was developed by Song et al. with the aim to increase the ocular drug resistance time and improve efficacy (Song et al. 2018). The gelation was performed first ionically, by using β-glycerophosphate disodium salt hydrate (β-GD) as crosslinker, followed by covalent crosslinking with genipin; a fast gel formation at 37 °C was observed. The feasibility of using this hydrogel as a topical eye-drop formulation for sustained release of timolol maleate was evaluated. In vitro performed test proved that the hydrogel was nontoxic to Chinese hamster fibroblast V79 cells and reduces the release rate of timolol maleate entrapped. The fast gel formation was observed after instilling the polymer solution into the lower conjunctival sac of the rabbit eyes, and the in situ formed hydrogels released the drugs in a sustained manner, suggesting that β-GD and genipin double crosslinking CS/G hydrogels could be a useful ocular drug delivery platform with enhanced therapeutic effects and reduced side effects.

Ionic and covalent crosslinked IPN-type microparticles based on CS and PVA were prepared in a simple water-in-oil emulsion by Cadinoiu et al. (2015). The presence of PVA influences the particle size and swelling capacity in different pH media. The ability to include and release pilocarpine is directly correlated with the swelling capacity, PVA content, and pH of the environment. The particles proved to

be biocompatible (nontoxic and hemocompatible), demonstrating their potential as ophthalmic drug delivery systems for pilocarpine.

Polymeric magnetic microparticles based on maghemite NPs dispersed in the same polymeric matrix (CS and PVA) were obtained by double crosslinking in reverse emulsion procedure, by using SO_4^{2-} as ionic crosslinker and GA as a covalent one (Balaita et al. 2015). The evaluation of their cytotoxicity and hemocompatibility has shown that they exhibit a prerequisite behavior for use in biomedical field, for drug targeting (i.e., 5-fluorouracil). Another type of magnetic NPs to target tumors was obtained from a maltose derivative of CS, by using the same method and same crosslinkers (Alupei et al. 2016).

Obtaining double crosslinked hydrogels from mixtures of CS and other polysaccharides, either for tissue engineering purposes or for drug inclusion, is reported in several recent papers. Each of the polysaccharides in the composition of the final hydrogel imprints several properties to the resulted hydrogel. Particles with relatively spherical morphology, macroporous, with an average diameter of about 2 mm, based on CS and alginate for the realization of inclusion and release systems of Dox were reported by Wu et al. (2020). In a first stage, the mixture of polymers is ionically crosslinked, by gelling with $CaCl_2$ of the caroxylate groups of alginate, after which the obtained particles (beads) are covalently crosslinked with GA (by participating in the reaction of the amino groups of CS). The cytotoxicity test and in vivo toxicity study showed that the hydrogel beads are relatively biocompatible. The swelling degree in water, porosity, and loading capacity with Dox increase with the increasing amount of the alginate in the composition of the particles. The obtained results recommend these hydrogel beads as a potential carrier for improving the colon targeting.

Alginate/CS microspheres were formed by an ionically double crosslinking technique, by an ionogelation mechanism followed by crosslinking. Firstly, alginate was crosslinked with Ca^{2+} ions, and further the CS was crosslinked with Na-TPP (Zhang et al. 2015a, b). Verapamil hydrochloride was loaded in the microspheres, and it appeared that this complex might be used as an effective carrier for sustaining the drug release.

Based on a mixture of poly(acrylamide) grafted carrageenan and sodium alginate, full-IPN type crosslinking hydrogel spherical beads were prepared by simple ionotropic gelation/covalent crosslinking method (Kulkarni et al. 2012). The beads exhibit pH-responsive behavior, evidently due to the polyanionic character of the polysaccharides. The ketoprofen (KTP) was loaded in the beads to obtain a system able to release the drug in the intestine, trans-passing the stomach barrier. Stomach histopathology of albino rats indicated that the gastric side effects like ulceration, hemorrhage, and erosion of gastric mucosa were reduced when the drug was loaded into these pH-responsive hydrogel beads.

kC-based particles mixed with another anionic polysaccharide, respectively gellan gum, were obtained by Kulkarni et al. firstly by ionic crosslinking with the SO_4^{2-} anion and then covalently with GA (Kulkarni et al. 2013a, b). Simvastatin (SMT) (a lipid-lowering drug used in the treatment of hypercholesterolemia, which decreases cholesterol levels by inhibiting the HMG-CoA reductase) was loaded in

these beads. It has been found that the drug is released faster only from ionically crosslinked particles whereas the covalent crosslinking prolonged the release. The in vivo tests performed on Wistar rats indicated that this system reduced the total cholesterol and triglycerides in the blood plasma effectively as compared to pure SMT.

Controlled release repaglinide loaded beads using sodium alginate and pectin, with double crosslinking for effective control of drug release, were recently reported (Awasthi et al. 2017). The presence of carboxylic groups in both polysaccharides makes it possible for their ionic crosslinking in a first stage, using $CaCl_2$ for this purpose. Their dimensional stabilization is subsequently done by chemical crosslinking with epichlorohydrin which results in the -OH groups on the backbone of the two polymers. The beads have an exceptionally good loading efficacy of repaglinide (up to 82.29%), being able to release the drug up to 61% in 12 h. The system is a good candidate for loading and release of drugs that are absorbed throughout the gastrointestinal tract, improving their bioavailability and enhancing the solubility.

Polymer crosslinking combined with ionic one is a double crosslinking coating possibility applicable to ionic polysaccharides, their derivatives, or mixtures with other polymers (natural or synthetic). It is applied to improve especially the mechanical properties of the gels or the stability of the particles. Some papers published in recent years report hydrogels obtained in this way, based on alginate. Dual-crosslinked particles based on methacrylated alginate obtained by W/O emulsion method were developed as intracellular delivery vehicles for the internalization and release of Dox (Fenn et al. 2016). Crosslinking was performed using light exposure in combination with ionic crosslinking by Ca^{2+} ions. While non-loaded microspheres were noncytotoxic to human lung epithelial carcinoma cells (A549s), Dox-loaded sub-microspheres significantly reduced mitochondrial activity after 5 days of culture. These photo-crosslinked particles may be a potential chemotherapeutic delivery system for cancer treatment. Also, alginate-MA was used to create hydrogels with different levels of crosslinking and subsequently different material properties by ionic and ultraviolet light crosslinking mechanisms (Samorezov et al. 2015). MC3T3 cells showed significantly enhanced proliferation on the surface of dual-crosslinked hydrogels compared with only calcium-crosslinked hydrogels. The alternation of the crosslinking mechanism permitted local regulation of the hydrogel physical properties and alignment of cells seeded on their surface, leading to spatial control over cell attachment and proliferation.

Methacrylated k-carrageenan can form hydrogels by double crosslinking. The methacrylate group introduced into the kC backbone enables the formation of chemically crosslinked gels via UV exposure; ionic crosslinking is performed later in the presence of KCl (Mihaila et al. 2013). The macrogel-type hydrogels can be applied in tissue engineering applications, having proper mechanical properties and ability to be loaded with cells (NIH-3T3 fibroblasts as model), and the swelling properties in aqueous environments are also recommended as potential supports for the inclusion/release of drugs. The same type of hydrogel has recently been reported

by Lim et al. (2020). The hydrogel shows a great potential as a bioink candidate that can be usefully applied to bioprinting-based tissue engineering applications.

Hydrogels based on marine polysaccharides can also be obtained by combining other crosslinking methods. One possibility is the association of *polymer crosslinking* with complexation using polyvalent metal ions. Recently, it has been reported to obtain a full-IPN hydrogel consisting of two CS derivatives, catechol-modified methacryloyl chitosan (CMC) and methacryloyl chitosan (MC), with high crosslinking-network density, with high compressive modulus and high ductility (Wang et al. 2019). The hydrogel can be made in situ after injection, the crosslinking being ensured by the photochemically initiated polymerization, as well as by the catechol-Fe^{3+} chelation which can covalently link with amino, thiol, and imidazole groups. The adhesive property of the hydrogels and hemostatic activity of CS led to a synergistic hemostatic performance in vivo. Biomedical applications of these hydrogels include wound areas, granulation tissue formation, angiogenesis, and collagen deposition, but there are also uses for drug release.

By combining physical and chemical crosslinking, hydrogels with superior mechanical properties, but also with high water absorption capacity, can be obtained, important properties for drug release applications. An interesting route to synthesize double crosslinked chitin hydrogels through a sequential chemical and physical crosslinking strategy was reported by Xu et al. (2016). The chemical crosslinking was realized by using ECH; subsequently, the hydrogels were immersed into a solution of aqueous ethanol (different concentrations) to form physically crosslinked domains through hydrogen bonding, hydrophobic interactions. These chitin hydrogels are expected to be promising candidate applications, such as in contact lenses, tissue engineering, and extracellular matrices requiring large-scale fabrication. Double covalent crosslinking is also a way to obtain hydrogels with various biomedical applications. A series of injectable in situ dual crosslinking of hyaluronic acid thiolated derivative (HA-SH) and oxidized alginate-based hydrogels were recently prepared by Zhang et al., through the covalent crosslinking between HA-SH and oxidized alginates via hydrazone and disulfide bonds (Zhang et al. 2019a, b). BSA was used as a drug model and incorporated into the hydrogels to evaluate the drug-controlled release property. The obtained results prove that these dual-crosslinking hydrogels could be used for drug delivery system, tissue engineering, and regenerative medicine.

2.5 Conclusions

The literature data presented in this chapter focused on progress concerning the preparation of new drug delivery systems based on marine polysaccharides. The tremendous amount of articles already published in this field clearly indicates that marine polysaccharides have an important practical interest in biomedical applications. They have been used especially as drug-loaded excipients in hydrogel formulations like films, beads, and micro-/nanoparticles, which were prepared by a wide range of crosslinking methods. However, their intrinsic characteristics, such as

biocompatibility, biodegradability, non-toxicity, stimuli responsiveness, etc., allow them to evolve considerably in the future. In addition, their facile chemical modifications are a considerable asset in developing new biomaterials which can be used in innovative applications. An important drawback is represented by the large use of potentially toxic crosslinking agents, such as glutaraldehyde. Therefore, further multidisciplinary investigations are necessary in order to develop novel polysaccharide derivatives which can be crosslinked in situ or without the use of toxic crosslinking agents.

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3 Chitosan and Chitooligosaccharides: Preparation, Characteristics, and Their Potential Application as Therapeutic Agents

Yusro Nuri Fawzya, Pujoyuwono Martosuyono, and Hari Eko Irianto

Abstract

Chitosan is one of the marine biomaterials widely found in crustacean, insect, arachnids, and fungi as the deacetylation product of chitin. Due to its biocompatibility, biodegradability, bioactivity, and nontoxicity, it has become of great interest and a good material for various applications in pharmaceutical and biomedical fields. The limited solubility of chitosan in acidic solution is then developed into various modification products, one of which is chitooligosaccharides (COS). This chapter overviews chitosan and COS including the preparation methods, properties, and several applications for biomedical purposes.

Keywords

Chitosan · Chitooligosaccharides · Preparation · Properties · Biomedical

3.1 Introduction

Chitosan is a natural polymer which can be derived through deacetylation process from chitin, a natural polysaccharide which is the second most abundant biopolymer after cellulose. Chitin is naturally found in the shells of fish, prawn, shrimp, krill, lobster, crab, cone snails, etc. which are being generated regularly as waste from fish industries and from household garbage. It can be obtained from insect cuticles, egg shells of nematodes and rotifers, the radulae of mollusks, cuticles of arthropods, pen and cuticle of squid, the diatom *Thalassiosira fluviatilis*, fungi, as well as filaments

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ejected by *Phaeocystis* in seaweed. Chitin, as structural polysaccharides, functions as supportive and protective material in the organisms (crustaceans, mollusks, insects, arachnids, and yeast or fungi) (Chawla et al. 2014; Dima et al. 2017; Firdous and Chakraborty 2017; Sani et al. 2017).

Some of the chitin-producing shrimps are *Penaeus monodon*, *P. indica*, *P. carinatus*, *Fenneropenaeus indicus*, and *Squilloides leptosquill.* The crabs that show chitosan release include *Portunus pelagicus*, *Sesarma plicatum*, and *Scylla serrata.* Chitins are also identified from white-rot fungus *Rigidoporus lignosus* as well as the mycelia, the caps, and stalks of fruiting bodies of four edible mushrooms, *Lentinus edodes*, *Lyophyllum shimeji*, *Caju*, and *Volvariella volvacea.* The exoskeleton of the short-horned grasshopper *(Schistocerca gregaria)* also produces chitin. Fungi and yeast releasing chitosan are *Aspergillus niger*, *A. terreus*, *Penicillium waksmanii*, *P. aurantiogriseum*, *P. viridicatum*, *P. citrinum*, and *Saccharomyces cerevisiae* (Ebrahimzadeh et al. 2013; Akila 2014; Krishnaveni and Ragunathan 2015; Sakthivel et al. 2015; Sartika et al. 2016; Firdous and Chakraborty 2017; Parthiban et al. 2017; Sani et al. 2017).

Of all chitin sources, crustaceans are the main source of chitin produced commercially, specifically from waste of shrimp or crab industries. The waste of crab, shrimp, and lobster shells globally reaches 6–8 million tons annually which is predicted 1.5 million tons in Southeast Asia alone (Yan and Chen 2015). The components proportion of commercial crustacean wastes based on dry weight is chitin 15–40%, protein 20–40%, and minerals 20–50% (Ruiz and Corrales 2017). Thus, chitin is produced by removing protein and minerals from the crustacean shells, which is commonly carried out by chemical method rather than enzymatic method. Deproteination of crustacean shells is conducted by using sodium hydroxide, while acid treatment with chloride acid, sulfuric acid, or nitric acid can be applied to remove minerals/demineralization. So far, preparation of chitin by enzymatic method is still limited for research purposes. Microbial proteases are employed to deproteinize crustacean shells, whereas lactic acid-producing bacteria are used for demineralization of the carapace of crustaceans. The lactic acid produced reacts with calcium carbonate or calcium phosphate yielding calcium lactate which is subsequently precipitated and eliminated. However, the quality of chitin produced is lower than that of produced by chemical method (Antonino et al. 2017; Ruiz and Corrales 2017). A reverse result is obtained from biologic chitin extraction using lactic acid bacteria and/or proteolytic bacteria which give more prospective results. The method produces a good quality of chitin and a high protein liquid fraction which can be used for human and animal feed, and other by-products, i.e., natural pigments, especially astaxanthin. Because of the low yield product compared to the chemical method, scope of the biologic method is still limited to laboratory scale (Arbia et al. 2013). Chitin is then deacetylated to produce chitosan which can be carried out by chemical as well as enzymatic methods.

Chitosan has a wide range of applications, including in agricultural, pharmaceutical, biomedical, and other various fields. The world chitosan market size is valued at $1.7 billion in 2019, and is projected to reach $4.7 billion by 2027, growing at a CAGR of 14.5% from 2020 to 2027. The main global producers of chitosan are

Japan, the USA, China, India, Australia, Poland, and Norway (Dima et al. 2017). Asia-Pacific accounted for the highest chitosan market share, owing to easy availability of crustacean waste in coastal areas (Allied Market Research 2020). The market size of chitosan for cosmetics/toiletries segment is projected to increase approaching to 18.5% in the coming years (Global Market Insights 2018).

Chitosan is insoluble in water and most organic solvents, which limits its applications in various fields. The low molecular weight chitosan, chitooligosaccharides (COS), and other derivative products of chitosan can be used to avoid this hurdle (Mourya et al. 2011). Various methods have been developed to modify chitosan in order to improve its properties through chemical/enzymatic methods to generate depolymerized and/or new derivatives. The production of COS and low molecular weight chitosan can be done by physical (such as sonication, irradiation, etc.), chemical (such as by using HONO, etc.), and enzymatic methods which involve specific enzymes (chitinolytic enzymes) or nonspecific enzymes (such as proteolytic, cellulolytic, lipolytic, amylolytic enzymes) (Baxter et al. 2005; Varun et al. 2017; Zhou et al. 2019).

Chitosan nanoparticles can be formed by the cross-linkers, i.e., STPP (sodium tripolyphosphate) by ionic bonds and glutaraldehyde by covalent bonds (Islam et al. 2019).The chemical modifications of chitosan to produce chitosan sulfate and carboxymethyl chitosan have been confirmed by FTIR and elemental analysis (Blanco et al. 2013).The chitosan derivatives can be obtained by chemical modification of the chitosan reactive functional group, in which the active groups $-OH$ and $-NH_2$ are susceptible to chemical reactions. New methods for reducing the molecular mass of chitosan are high-pressure homogenization, plasma, or using zeolites adsorbents to purify acid hydrolysis COS and low molecular weight chitosan (Brasselet et al. 2019; Wang et al. 2020).

Other than chitosan's ability to induce erythrocyte aggregation, promote platelet activation, and activate complement systems, modified chitosan derivatives have improved biocompatibility, biodegradability, bioactivity, and nontoxicity while retaining the original antibacterial, bactericidal, anticancer, and antiviral pharmacological effects, such as the ability to bring about erythrocyte aggregation, platelet activation, and complement system activation (Wang et al. 2020). These improved properties expand the range of uses for chitosan, including medical and pharmaceuticals, water treatment, agrochemicals, as well as food and beverage. Chitosan is employed in water treatment to adsorb metals, harmful compounds, iron, and other pollutants from wastewater due to its adsorption property. Chitosan is an effective, inexpensive, and environmentally friendly technique to treat water. Chitosan is used as a preservative, nutritional supplement, and anticholesterol ingredient in the food and beverage business. Chitosan is utilized in cosmetics for hair and skin care. Because of its bioactivity, antibacterial activity, and antioxidative activity, chitosan and its derivatives are useful in a range of medical and pharmaceutical applications. Chitosan is applied to wound ointments and medical device coatings. Chitosan is utilized in contact lenses and in medical applications as a drug delivery agent (Markets and Markets 2018). Tissue engineering (Pandey et al. 2017), drug delivery systems (Mengatto et al. 2012), gene therapy systems

(Kritchenkov et al. 2017), wound healing (Patrulea et al. 2015), antioxidant (Rajalakshmi et al. 2014), and antibacterial (Goy et al. 2009) are some of the potential therapeutic applications of chitosan.

In this chapter chitosan with regular and nanoparticle sizes as well as its depolymerized product will be discussed, including their preparation methods and their characteristics. Then, the most recent application of chitosan as therapeutic agents will be overviewed, focusing in tissue engineering, drug delivery system, gene therapy system, and antimicrobial materials.

3.2 Chitosan and Nanochitosan

Chitosan is a natural polymer composed of D-glucosamine and N-acetyl-D-glucosamine linked through β-1,4 glycosidic bonds, generated from partial or total chitin deacetylation. Chitin is an intermediate product prior to chitosan, a poly-(β-(1 → 4)-N-acetyl-D-glucosamine, the main component of crustacean and other arthropod exoskeleton. Chitin is naturally available in three different forms depending on the source designated as α-, β-, and γ-chitin. Each form has a specific structural manner related to the chain arrangement. α-Chitin is the most stable form and built of antiparallel chains, usually found in the exoskeleton of crustaceans and fungi (Jung and Zhao 2013; Alvarez 2014). β-Chitin, composed of parallel chains with a weaker hydrogen bonding, can be obtained from squid pens (Jung and Zhao 2013; Alvarez 2014). γ-Chitin contains both types of parallel and antiparallel chains, which is typically found in insects and the *Loligo* squid stomach (Jang et al. 2004). Commercially, chitin is extracted from crustacean waste including shrimp and crab shells which is categorized as α-chitin.

So far, chitin and chitosan have a great interest for numerous researches due to not only its wide range applications but also its unique characteristics offering chitosan considerable usage in various biomedical applications.

3.2.1 Preparation of Chitosan and Nanochitosan (Chitosan Nanoparticles)

Chitosan can be prepared by chemical and biotransformation via enzymatic method. Even though chemical method consumes much energy, is harsh, and has higher risk of environmental pollution, this method is used extensively for industrial processing of chitosan. It is because of the fast and easier process compared to enzymatic method. The advantages of biological production process of chitosan are particularly in producing higher molecular weight of chitosan with the desired degree of polymerization (Tsigos et al. 2000).

3.2.1.1 Chemical Method

Most commercial chitosan is prepared from crustacean shells due to the availability of the raw materials. Basically, chitosan is produced mainly by elimination of protein

(deproteinization), minerals (demineralization), and acetyl group (deacetylation), where the first and second step can be reversed in sequence. From the first two steps, chitin will be produced, and the next step is deacetylation of chitin to obtain chitosan as the final product.

Various chemicals can be employed for shell deproteinization, including NaOH, Na_2CO_3, $NaHCO_3$, Na_2SO_3, $NaHSO_3$, Na_3PO_4, Na_2S, KOH, K_2CO_3, $Ca(OH)_2$, and $CaHSO_3$ (Younes and Rinaudo 2015). However, the most popular reagent employed is dilute NaOH (0.5–1.25 M) solution with the reaction temperature at 65–100 °C for 0.5 to 72 h. The use of this chemical at high temperature causes depolymerization which may influence chitin properties related to viscosity, molecular weight, as well as degree of acetylation (Gortari and Hours 2013; Antonino et al. 2017; Pighinelli 2019). Removing minerals (mostly $CaCO_3$) from the deproteinized crustacean shell is possibly performed by HCl, HNO_3, H_2SO_4, and CH_3COOH, but so far dilute HCl solutions at room temperature are the most desirable to be applied for demineralization. The reaction is employed at room temperature, which usually takes 2–3 h under stirring (Younes and Rinaudo 2015). Exception methods may be done, such as the deproteinization of crab shell and squid pen using 4% NaOH at lower temperature (10 °C) (Jang et al. 2004) and the use of ethylenediaminetetraacetic acid (EDTA) or acetic acid to reduce degradation in demineralization process (Younes and Rinaudo 2015).

Deacetylation is a process of elimination of acetyl group from chitin molecules. Chitin has been categorized as chitosan when the degree of deacetylation (DD) of chitin reaches more than 50% (Younes and Rinaudo 2015). Deacetylation is generally performed by using concentrated sodium hydroxide solution (approx. 50–70%) at high temperature (80–110 °C) for few hours. Higher concentration of NaOH and higher temperature process of deacetylation will produce chitosan with higher degree of deacetylation. However, depolymerization possibly occurred which is showed by decreasing of chitosan molecular weight. Variation of NaOH concentration, temperature, as well as time of deacetylation process will produce chitosan with various DD and various chitosan properties especially viscosity and molecular weight (Galed et al. 2008; Pires et al. 2014; Younes and Rinaudo 2015). Raw materials as chitosan sources also determine type and characteristics of chitosan produced.

Nanochitosan or chitosan nanoparticle is a modified chitosan form prepared by reducing its particle size producing chitosan in nanosize. This modification is purposed to improve the chitosan properties due to the much smaller size and large surface area that make it easier to diffuse or adsorb into targeted substances in its application. In addition, when functioning as a pharmaceutical carrier, delivery and uptake by cells are more efficient (Othman et al. 2018).

Many studies and reviews showed the potential of nanochitosan in various applications, such as antimicrobial (Yang et al. 2010; Aliasghari et al. 2016; Abdeltwab et al. 2019), drug delivery system (Elgadir et al. 2015; Li et al. 2018), and tissue engineering (Gokila et al. 2018; Sangeetha et al. 2019).

The characteristics of chitosan that have been used for the manufacture of chitosan nanoparticles vary, including chitosan with molecular weight (MW) of

21 kDa and DD 87% (Su et al. 2010), MW 360 kDa and DD 95% (Wu et al. 2005), MW 100 kDa and DD 80% (Kim et al. 2006), and viscosity 95 mPa and DD 86% (Grenha et al. 2005). Meanwhile, the commercial chitosan traded has an average MW and DD in the range of 3800 to 20,000 daltons and 66 to 95%, respectively (Agnihotri et al. 2004).

Chitosan nanoparticles can be prepared by several methods, such as ionic or ionotropic gelation, complex coacervation, coprecipitation, microemulsion, emulsification solvent diffusion, emulsion-based solvent evaporation, and reverse micellar method (Mohammed et al. 2017). However, the most common method applied is ionic gelation method, which is a simple technique. In this method, the positive charge of chitosan solution in acetic acid is added with any polyanionic solution, such as sodium tripolyphosphate with negative charge (with or without a stabilizing agent). The interaction of the two charges results in separation of chitosan in spherical nanoparticles (Yang et al. 2010; Mohammed et al. 2017; Sangeetha et al. 2019).

Practically, 2% acetic acid solution is generally used to dissolve chitosan, and approximately 0.8% STPP solution is then added dropwise while stirred continuously for 30 to 120 min at room temperature. The nanochitosan formed is separated by decantation or centrifugation and then prepared for drying. Several factors affect the physical characteristics of nanochitosan including concentration of chitosan and STPP solution, pH, temperature and stirring speed, number of cross-linking, and molecular weight of chitosan (Gopal and Nandakumar 2015; Othman et al. 2018). Improvement of chitosan nanoparticles properties can be provided by optimization process through reducing concentration of acetic acid at ambient temperature. These treatments result in good storage stability and produce monodisperse and low molecular weight nanochitosan. The stability is related to the more hydrogen bond of chitosan interacting with water molecules due to the fast cooling rate of suspension at low temperature (Fan et al. 2012). A modification process can be employed to improve the chitosan nanoparticles properties by encapsulating it using the right compounds. L-Ascorbic acid (LAA) as hydrophilic antioxidant and thymoquinone as hydrophobic antioxidant can encapsulate chitosan nanoparticles resulting in improvement of their pharmaceutical efficiency (Othman et al. 2018). Firstly, the LAA is added into tripolyphosphate (TPP) solution; then the mixture is reacted with the chitosan as previously described in ionic gelation method producing nanochitosan-LAA, whereas the addition of thymoquinone is conducted by dissolving the thymoquinone in dimethylsulfoxide (DMSO) and adding the solution to chitosan. Then, TPP as negatively charged is poured into the mixture of chitosan-thymoquinone providing nanochitosan-thymoquinone (Othman et al. 2018). Other cross-linker used in ionic interaction with chitosan is sodium sulfate. The cross-linking of low molecular weight chitosan with sulfate anion as cross-linking bridge producing low molecular weight chitosan sulfate nanoparticles which is relatively stable and considered appropriate carrier in drug delivery application (Al-Remawi 2012). The size of nanochitosan produced by this method ranges from 20 to 200 nm and 500 to 900 nm (Mohammed et al. 2017).

The method of complex coacervation or polyelectrolyte complex is conducted by separation of nanoparticles through mixing electrostatically driven liquids, such as the production of DNA-chitosan nanoparticles. An ionic solution of dextran sulfate DNA solution interacts with the cationic chitosan solution which is dissolved in acetic acid, gelatin, and polyethylenimine followed by charge neutralization generating nanoparticles in the size of 300–500 nm (Hembram et al. 2014; Mohammed et al. 2017). This method has an advantage such that the process can be taken place in an aqueous solution at low temperature (Mohammed et al. 2017). The coprecipitation method to produce chitosan nanoparticles can be prepared by addition of acidic chitosan solution to alkaline solution (pH 8.5–9.0) such as ammonium hydroxide generating spherical and uniformly distributed nanochitosan (Hembram et al. 2014; Mohammed et al. 2017). Preparation of chitosan nanoparticles by microemulsion method is carried out by using surfactant dissolved in n-hexane which is then added with chitosan and glutaraldehyde steadily stirred at ambient temperature (Hembram et al. 2014).

3.2.1.2 Enzymatic Method

Enzymatic deacetylation of chitin into chitosan can be achieved by chitin deacetylases. The most important advantage of enzymatic reaction compared to chemical processes is the product of chitosan with high molecular weight and certain degree of deacetylation (Tsigos et al. 2000). Chitin deacetylases have been found in several fungi, insects, and marine bacteria, particularly those which have chitin and chitosan as a structural component of their cell wall. The enzyme will be secreted to the periplasmic compartment of cell as extracellular enzyme and contribute to biosynthesis of chitosan (Tsigos et al. 2000; Zhao et al. 2010). Preparation of chitosan by enzymatic method using chitin deacetylases should employ soluble forms of chitin such as glycol chitin and chitosan of variable DD, because the enzymes are hard to access toward crystalline chitin (Grifoll-Romero et al. 2018). Pretreatment of chitin may be applied to enhance the accessibility of the enzyme to the acetyl group, such as by partially deacetylated chitin substrates. The use of chitin deacetylase from *Bacillus* K29-14 to produce chitosan is preceded by soaking chitin in 60% NaOH solution at 60–75^{o}C for 60–180 min bringing about chitin swelling and allowing enzyme access to acetyl group of chitin. Chitosan produced has DD of 72–99% with lower molecular weight indicating the depolymerization occurred during the process (Emmawati et al. 2007).

There are other enzymes involved in chitin and chitosan conversion, such as chitinase and chitosanase which catalyze the hydrolysis of glycosidic bonds in different substrate specificity (Jaworska 2012). The products of enzymatic deacetylation can be further modified enzymatically by COS deacetylases to generate products with desired chain arrangement (Hirano et al. 2017). All of these enzymes are widely used as useful tools toward production of chitosan and COS biotechnologically, especially in a controlled, well-defined, and non-degradative process (Hamre et al. 2015). However, the enzymatic methods have main limitation including the application of multistep character of the process and high price of

enzyme, which makes them undesirable options from an economic point of view (Kim and Rajapakse 2005)

3.2.2 Characteristics of Chitosan

Chitosan has lots of special characteristics including physicochemical properties due to its important role of functional group (amino and hydroxyl). The free amino group gives chitosan a positive charge and determines the degree of deacetylation which the latter then affects greatly on the crystallinity of chitosan that is associated with its solubility. The deacetylation requirement is usually $\geq$70% for chitosan which is commonly acceptable for various applications. The number of functional group of chitosan is influenced by preparation method involving concentration of chemical used, temperature, preparation time, etc. which can impact on chitosan properties and functionality such as molecular weight, viscosity, the ability on heavy metal chelation, as well as coagulation of fat and proteinaceous materials (Chi and Cheng 2006; Younes and Rinaudo 2015). Decolorization treatment can also reduce the quality of chitosan such as producing a lower viscosity chitosan which is related to changes in molecular weight. The biological properties of chitosan are widely applied to the development of biomedical products such as antibacterial, wound healing, drug delivery, etc. Quality and different sources of raw material also confer an important effect on chitosan characteristics. The use of shrimp waste produces chitosan with higher viscosity and solubility (5300 cPs and 97%) compared to *Squilla* and crab shell waste chitosan, which have viscosity of 2600 cPs and 465 cPs and the solubility of 89.7% and 85%, respectively (Parthiban et al. 2017).

Molecular weight (MW) and viscosity of commercial chitosan are usually classified into five groups, i.e., ultralow MW (<20,000 Da) or viscosity of <30 cPs; low MW (20,000–250,000 Da) or viscosity of 30–300 cPs; medium MW (250,000–1250,000 Da) or viscosity of 300–1000 cPs; high MW (1250,000–1,800,000 Da) or viscosity of 1000–2000 cPs; and very high MW (>1,800,000 Da) or viscosity of 2000–3500 cPs. The viscosity of chitosan is proportional to the concentration. Chitosan viscosity of 1% in 1% acetic acid is usually used in determining the specification of chitosan or its derivatives. Chitosan with low molecular weight and viscosity can be obtained when deacetylation processes employ higher concentration of NaOH, higher temperature, and longer time. Further impact will occur on increasing DD value and solubility.

Chitosan is a cationic polyelectrolyte, white in color, and solid shape. It is soluble in weakly aqueous acidic solution, such as acetic acid, formic acid, and lactic acids, but insoluble in water at neutral and alkaline solutions as well as organic solvents. The degree of solubility is much affected by the DD value which is correlated with the free amino group and distribution of the acetyl group along the chains of chitosan molecule. The free amino group is the most important factor in determining the chemical and biochemical reactivity of chitosan (Younes and Rinaudo 2015; Majekodunmi 2016; Zhang et al. 2016; Parthiban et al. 2017). Chitosan is also

Table 3.1 Specification for commercial chitosan[a]

Quality parameters	Specification		
	Pharmaceutical grade	Food grade	Industrial grade
Appearance	White or light yellow, powder or flake	White or light yellow, powder or flake	White or light yellow, powder or flake
Degree of deacetylation	70–100	70–100	70–100
Viscosity	≤5 cPs	≤5 cPs	50–500 cPs
Moisture content	<10.0%	<10%	<12%
Residue on ignition	<0.2%	<0.2%	<0.5%
Insolubles	<1.0% insolubility	<1.0% insolubility	<1.0% insolubility
Heavy metal (Pb)	<10 ppm	<10 ppm	–
As	<10 ppm	<10 ppm	–
Total bacterial count	≤1000 cfu/g	≤1000 cfu/g	–
Odor	No taste and smell	No taste and smell	–
pH	7–9	7–9	7–9

[a]Summarized from several commercial chitosan requirements

biodegradable by chitosanase as a specific enzyme or by nonspecific enzymes such as cellulases, proteases, and lipases producing COS which are water-soluble.

The degree of deacetylation value is correlated with the amount of protonated amino groups in chitosan molecule. The higher the DD is, the higher the chitosan purity from the impurities of protein, minerals, pigments, and acetyl group which is accompanied with its higher solubility in acidic solution. The chitosan purity is used as the basis for the classification of commercial chitosan into pharmaceutical grade, food grade, and industrial/technical grade (Table 3.1). Other properties of chitosan that are commercially relevant are its biocompatibility, biodegradability, and transformation capacity into fibers, gels, beads, or other forms that are suitable for foods, cosmetics, and pharmaceutical applications (Majekodunmi 2016). Even though the requirement of chitosan for pharmaceutical is similar to food purposes, practically it is different in the purity and DD value which are higher for pharmaceutical application, usually above 90%. Purity of chitosan is also determined by wide range of chitosan sources and various processing techniques leaving residual or impurities of protein, ash, or microbiological contaminants. Variation in requirements of chitosan specification of each grade is possible due to the development of chitosan modification and the differences of method employed for analyses.

Degree of deacetylation of chitosan also affects the shelf life of chitosan during storage. A higher DD (>85%) is allowed in slower degradation process with a low degradation index in the aqueous conditions. On the contrary, chitosan with a lower DD (82–65%) would have a shorter shelf life due to the faster degradation (Rodríguez-Vázquez et al. 2015). However, chitosan stability is influenced by

internal and external factors. Internal factors include molecular weight, purity level, DD, polydispersity index, and moisture content. Environment conditions, such as temperature and humidity of storage room, and processing condition, including physical methods, sterilization, or other thermal processing, are external factors that affect the degradation rate of chitosan during storage (Szymańska and Winnicka 2015).

Similar to chitosan, chitosan nanoparticles are biocompatible and biodegradable, while its bioactivities are beneficial to wide range of applications, including for pharmaceutical and medical purposes. Chitosan nanoparticles prepared by the aggregation method of chitosan exhibit the same chemical structures and homogenous surface with the native chitosan, but reduce in crystallinity and increase in the amorphous region, improving its hydrophilic properties. The nanochitosan has greater thermal stability (Jampafuang et al. 2019; Guimarães et al. 2020). The particle size of chitosan nanoparticles has a positive correlation with DD of chitosan which is probably related to the availability of amine groups in the chitosan molecules. Chitosan with higher DD can be a stronger protonation of the amine group which makes the chitosan to expand larger generating a larger particle size (Jampafuang et al. 2019).

3.3 Chitooligosaccharides (COS)

COS, or chitooligomers or chitosan oligomers, are degradation products of chitosan with degree of polymerization $\leq$20 and average molecular weight <5 kDa (Jung and Park 2014; Guo et al. 2018). Compared to chitin and chitosan, the low molecular weight COS has advantageous properties such as high solubility, good biocompatibility, good water absorption, etc. which support on its biological properties. Several technological approaches can be used to produce COS including chemical, enzymatic, physical, and electrochemical approaches of chitosan degradation (Liang et al. 2018). However, commercial production of COS is commonly prepared through chemical and enzymatic methods.

3.3.1 Preparation of COS

3.3.1.1 Chemical Method

Preparation of COS by chemical methods includes hydrolysis reaction using acids or oxidative reaction using hydrogen peroxide. The (1 → 4)-β-glycosidic bonds in the chitosan molecule are cleaved randomly during the hydrolysis process, producing homo- or hetero-oligosaccharides depending on the presence of acetyl group in the chitosan molecule. The process is relatively simple and efficient, but it is difficult to control and sometimes produces various by-products in which the COS is not easy to separate and purify. The chemical degradation method is also environmentally hazardous and produces oligomers with various degrees of polymerization (DP) and degrees of acetylation (DA).

Concentrated HCl (12M) employed to produce COS via hydrolyzing fully N-deacetylated chitosan at 72 °C for 75 min is an optimum condition to produce glucosamine oligomers with DP of 1–15 (Cartier and Domard 1989). Similar to those results, degradation of chitosan by chloride acid method results in hydrolysates with DP up to 16 and more monoacetylated residues than with enzymatic methods (Cabrera and Van Cutsem 2005).The use of dilute HCl in the presence of zeolite is developed to prepare COS through the technique of adsorption reaction (Ibrahim et al. 2016). Firstly, chitosan is mixed with deionized water and then added with zeolite and HCl followed by heating the mixture under reflux at 100 °C with stirring for 2 h. After cooling to room temperature, the mixture is centrifuged. The solid fraction is then added with distilled water, vortexed, and centrifuged. The supernatant, as chitooligomer, is then evaporated. Another acid employed to depolymerize chitosan in the preparation of COS is nitrous acid (HONO). Chitosan solution (in dilute acetic acid) is added with $NaNO_2$ and kept overnight at 4 °C or room temperature (Tømmeraas et al. 2001; Varun et al. 2017). The proportion of $NaNO_2$ depends on degree of acetylation (DA) of chitosan used. The higher the DA of chitosan, the higher the proportion of $NaNO_2$. Another chemical method used in COS preparation is oxidative degradation method using oxidants such as hydrogen peroxide. A random degradation of partially deacetylated chitin and chitosan occurred by the addition of hydrogen peroxide at low concentration, and reducing their molecular weight followed the first-order kinetics (Chang et al. 2001). The formation of glucosamine and COS depends on the physicochemical characteristics of chitin and chitosan, reaction temperature, and the peroxide concentration. Degradation of chitosan by the use of 4.5% hydrogen peroxide at 56°C for 6 h produces COS with a degree of polymerization below 6 (Wu et al. 2017). Application of 1% hydrogen peroxide to 3% chitosan solution (in dilute lactic acid) at ambient temperature obtains COS with molecular weight of 4500 g/mol at 15 days of the reaction period (Hai et al. 2019).

3.3.1.2 Enzymatic Method

Enzymatic degradation is of great interest to produce COS with the molecular weight distribution controlled and without any modifications (Lodhi et al. 2014; Liang et al. 2018). In addition, the method offers an eco-friendly and controllable process compared to chemical method which is harsh and needs more purification steps to reduce the residual strong acid. However, this method has limited application due to the cost, availability, and specificity of chitosanases as specific enzymes for COS production (Vishu Kumar et al. 2004). Chitosanase belongs to chitinolytic enzymes which are classified as specific enzymes to produce COS via hydrolyzing chitosan. The enzymes catalyze the cleavage of β-1,4-glycosidic linkages in chitin and chitosan, while the other enzymes referred as nonspecific enzyme group (protease, cellulase, lysozyme, lipase, amylase, etc.) also can be used in the preparation of COS. Most chitosanases employed in the COS production have been found in a variety of microorganisms, including bacteria and fungi. So far their commercial availability is limited. The product of enzymatic hydrolysis is chitosan hydrolysates which are mixtures of COS with DP of 1–7 depending on the reaction condition.

Prolonged hydrolysis period commonly produces monomer as the main product (Jung and Park 2014).

A unique endo-chitosanase from marine bacterium *Pseudoalteromonas* sp. SY39 that is both thermotolerant and cold-adapted enzyme produces chitodisaccharide as the major product after being applied to chitosan (Zhou et al. 2019). Chitobiose having the most proportion of hydrolyzed product is also produced from chitosan catalyzed by purified chitosanase from *Bacillus* sp. HW-002 at 40 °C for 5 h (Lee et al. 1996). High proportion of chitotriose to chitooctose in the COS is produced from chitosan by using crude chitosanase from *Bacillus cereus* P16 incubated at 37 °C for 24 h (Jo et al. 2003). The COS composition is affected by chitosan concentration. The higher the concentration, the higher DP of COS is produced. An exo-chitosanase, exo-chitobiohydrolase, produced by *Gongronella butleri* hydrolyzes soluble chitosan and glucosamine (GlcN) oligomers (DP > 4) producing chitodisaccharide $(GlcN)_2$ from the nonreducing ends of chitosan. The enzyme has the ability to digest the linkage of GlcN-GlcNAc, GlcNAc-GlcN, and GLcN-GlcN (Seki et al. 2019). The quality of both chitosan and enzyme used affects the quality or properties of COS produced. The use of low DD chitosan and crude chitosanase with low activity produces COS with high ash content and should be desalinated to obtain better properties of COS. This problem may be occurred in scaling up production of COS using crude enzyme from wild-type bacteria in which its activity tends to decrease during storage (Fawzya et al. 2018).

The use of nonspecific enzymes to hydrolyze chitosan has shown the ability of the enzymes to produce various COS. They include protease, cellulase, lipase, etc. The use of papain (0.003% w/w) in 16-h hydrolysis of chitosan DD 90% shows the highest molecular weight reduction compared to lysozyme and cellulase, producing COS with the average MW of 4.3 kDa (Laokuldilok et al. 2017), whereas the most effective enzyme in reducing MW of chitosan DD 80% is shown by lysozyme. Papain is predicted working better in cleaving the β-1,4-glucosidic linkage between two glucosamine molecules than cellulase and lysozyme, while lysozyme is more effective in cleaving the bond connecting glucosamine and N-acetylglucosamine molecules. The effectiveness of papain and lysozyme in the depolymerization of chitosan is also reported (Lin et al. 2009). However, the hydrolysis process is generally affected by type of enzyme, hydrolysis time, degree of acetylation, and initial molecular weight of chitosan (Laokuldilok et al. 2017).

The discovery of lytic polysaccharide monooxygenases (LPMOs) since the last 10 years has been a great interest of studies on COS production by using this enzyme to cleave the glycosidic bonds of crystalline chitin/chitosan. The enzyme works by oxidizing either C1 or C4 of the glucopyranose ring through an oxidative mechanism which needs a reductant, an oxygen containing co-substrates and a copper ion with a single bound. The reaction produces oxidized chain ends which then support chitinases/chitosanases in further depolymerization of chitin/chitosan (Vaaje-Kolstad et al. 2010; Bissaro et al. 2017). Some microorganisms producing LPMOs are reported, such as *Serratia marcescens* (Vaaje-Kolstad et al. 2010),*Bacillus cereus* (Mutahir et al. 2018), and *Vibrio cholerae* (Loose et al. 2014).

The synergy between LPMOs and chitinase for degradation of shrimp shell chitin is demonstrated by reacting the mixture of 10 mg/mL chitin, 1 mM ascorbic acid, and 20 mM Bis-Tris buffer (pH 6) with 0.5 μM LPMO (from *B. cereus* or *S. marcescens*) and 0.25 M GH18 chitinases, followed by degradation of the soluble fraction produced from the incubation time (up to 48 h) using 1.0 μM chitobiase for 24 h (Mutahir et al. 2018). The yield of products prepared by both enzymes simultaneously is 26% higher than the sum of the yields processed by the individual enzyme (LPMO and chitinase). In addition, GlcNAc produced corresponds with the yields of oxidized products. The solubilized oxidized products will increase correlated with solubilization of oxidized sites in the solid substrate due to the addition of chitinases to LPMO-pretreated chitin.

3.3.1.3 Physical Method

Degradation of chitosan by physical method usually produces COS with less contamination and low productivity (Liang et al. 2018). In addition, this method, such as ultrasonic, microwave, or gamma irradiation, is much simpler to keep the purity. The use of high-intensity ultrasonication on the chitosan solution (in acetic acid) decreases the intrinsic viscosity which is affected by the intensity and time of ultrasonication, thus resulting in lower molecular weight of chitosan (Baxter et al. 2005). The higher the intensity and the longer the time of sonication, the lower the viscosity as well as the molecular weight of chitosan. The initial molecular weight and viscosity of 1% chitosan (DD 81%) solution which are 867 kDa and 3.85 dL/g, respectively, decreased to 140 kDa and 0.75 dL/g at 35.2 W/cm^2 (power 10) sonication for 30 min. Preparation of COS can be carried out by irradiation of chitosan with 2–200 kGy doses of Co-60 gamma rays (Choi et al. 2002). Irradiation more than 100 kGy produces COS with darker brown color. At the 100 kGy dose of irradiation, the COS produced are mainly dimer, trimer, and tetramer with the proportion of 3.8, 3.9, and 2.5% in 100 cP chitosan solution, respectively. Degradation of chitosan solution by ultrasonic irradiation at frequency of 20 kHz is performed using different chitosan concentration, irradiation period, ultrasound power, and solution temperature (Kasaai et al. 2008). Depolymerization increases with the increase of ultrasound power, irradiation time, and solution temperature, as well as the decrease of chitosan concentration. A hydrodynamic cavitation may be an alternative method to degrade chitosan by passing the chitosan solution through a closed system constructed using an orifice plate. The degradation effect is better when using orifice plates with much more holes and smaller hole diameter increasing the intensity of cavitation. In addition, the degradation of chitosan increases with the increasing upstream pressure and at higher pH solution under acidic condition but decreases by increasing initial concentration of chitosan solution. This procedure is considered as prospective method due to the high degradation rate, low energy used, the availability of equipment, being safe to environment, a lot of samples used, etc. (Huang et al. 2013).

3.3.1.4 Electrochemical Method

Electrochemical method using Ti/Sb-SnO_2 electrode has a good efficiency in the degradation of chitosan (Gu et al. 2013). The electrode as non-active electrode performs better and has lower cost compared to Ti/TiO_2-RuO_2 electrode (Cai et al. 2011), due to the higher oxygen evolution potential of Ti/Sb-SnO_2 electrode. The molecular weight of chitosan rapidly decreases with time of electrolysis from 479 to 85 and 46 kDa (the reduction of 60.5 and 90.4%) at 30 and 60 min, respectively. However, some disadvantages of the method such as easy failure and short electrode endurance may restrict the use of this method (Liang et al. 2018).

3.3.2 Characteristics of Chitooligosaccharides (COS)

COS are derivatives of chitosan which have lower molecular weight. The characteristics of COS are much affected by their chemical properties. Like chitosan, COS is biodegradable and biocompatible. In addition, their greater solubility at neutral pH, low viscosity, and nontoxicity as well as their low molecular weight are beneficial for wider applications than chitosan, especially for food and biomedical applications (Park et al. 2011; Lodhi et al. 2014; Marmouzi et al. 2019). Their various biological activities, including antitumor, anti-oxidative stress, anti-inflammation, anticholesterol, antihypertensive, controlling arthritis, and increasing of calcium uptake, are also the reason of COS application in pharmaceutical and biomedical fields (Park et al. 2011; Lodhi et al. 2014; Marmouzi et al. 2019). Their molecular weight is usually less than 10,000 Da depending on degradation method (chemical, physical, enzymatic) and process condition. The COS also has degree of polymerization (DP) of less than 50 (Marmouzi et al. 2019). It is water-soluble but not soluble in acetone, ethyl acetate, and 2-4 alkyl alcohol. The solubility in methanol is shown by oligomers with DP of 2–4. On the contrary, oligomers with DP >5 are less soluble (Mourya et al. 2011). COS with shorter chain lengths have number of reducing units, and therefore the aldehyde groups in COS allow to react with amino groups as Schiff base formation, Maillard reaction, and Amadori rearrangement, generating product with undesirable characteristics such as brownish color, reduced water solubility, decreased thermal stability, diminished moisture-adsorption capacity, and physiological activity losses to COS (Mourya et al. 2011).

Composition of COS will determine their physicochemical properties which are much affected by methods of chitosan depolymerization and will further influence their bioactivities. A single-step enzymatic method using chitosanase compared to a two-step chemical-enzymatic hydrolysis is applied to COS preparation. The COS produced from the first method consists of lower proportion of fully deacetylated oligomers (42%) and higher monoacetylated oligomers (54%) compared to the second method (50% and 27%, respectively). The end product from the single-step enzymatic method contributes in stabilizing the lipopolysaccharides (LPS)-COS complex and has anti-inflammatory effect on both in vitro (using RAW264.7 macrophages) and in vivo (using LPS-induced mice) assays (Sánchez et al. 2018).

Effect of different enzymes applied to hydrolysis chitosan for COS preparation shows that lipase is the most effective for producing COS with the maximum FRAP (5.66 μmol TE/g) and ABTS (322.68 μmol TE/g) radical scavenging activity compared to amylase and pepsin. The COS from squid pen produced by lipase has water solubility of 49%, intrinsic viscosity of 0.41 dL/g, and average molecular weight of 79,000 Da. Addition of this COS with the concentration of 1% retards lipid oxidation of surimi gel kept at 4 °C, thus extending the storage life (Singh et al. 2019).

The adsorption of COS on activated charcoal increases with the smaller particle size of the charcoal, and its capacity reaches a maximum at pH 8–9 with no significant difference at various temperatures. The adsorption equilibrium is reached after 60 min, and the kinetic process is in accordance with the pseudo-second-order model and shows chemisorption properties. These data and information are important for the production and purification of COS using activated charcoal (Yu and Li 2019).

3.4 Potential Application of Chitosan and Its Derivatives as Therapeutic Agents

3.4.1 Tissue Engineering

Tissue engineering is a part of the regenerative medicine that involves materials (natural and/or synthetic polymers) to develop biomimetic scaffolds for repairing and regenerating tissue and, further, improving function on defective tissue. The biocompatibility, biodegradation, and non-cytotoxicity characteristics of biopolymers make them potential for use in regenerative medicine, and are considered in the selection of appropriate biomaterials which is a critical point in designing scaffolds for tissue engineering (Sultankulov et al. 2019). Chitosan is a natural biopolymer having the characteristics mentioned above, with other important features for scaffold specification including its physicochemical properties (solubility, molecular weight, water absorption capacity, porosity, mechanical and biological properties, etc.). In addition, chitosan molecule can be chemically modified to obtain additional functional properties which are promising to the application for scaffold construction (Rodríguez-Vázquez et al. 2015; Sultankulov et al. 2019).

Molecular weight is associated proportionally with viscosity. High molecular weight chitosan acts as a strong viscosity building agent when it is dissolved in acid solution and transforms into pseudoplastic material which is an important characteristic in tissue interaction. Porosity is an important feature for scaffolding due to the physical structure of the scaffold which should control cell functions by regulating the transfer of nutrients and other substances as well as water diffusion. Chitosan has been proven to generate a porous scaffold which can retain water and bioactive protein (Madihally and Matthew 1999; Rodríguez-Vázquez et al. 2015). The porous chitosan can be developed by freezing and lyophilization of chitosan solution under controlled condition. The hydrated porous chitosan membranes give at least twice

more surface area than nonporous chitosan membranes, but their elasticity and tensile strengths are estimated ten times lower than nonporous one (Madihally and Matthew 1999). The use of chitosan in tissue engineering is clinically safe for humans due to the biocompatibility property of chitosan and the fact that chitosan is composed by GlcN and GlcNAc, natural substances of mammalian tissue (Rodríguez-Vázquez et al. 2015). The cytocompatibility of chitosan film toward keratinocytes and fibroblasts with the cell proliferation is affected by the DD of chitosan (Chatelet et al. 2001).

Various forms of chitosan can be employed to construct scaffold, including bulk, microparticle, nano-fiber, hydrogel, and blended chitosan, with other natural or synthetic polymers. Natural polymers that can be combined with chitosan include collagen, gelatin, silk fibroin, hyaluronic acid, alginate, and cellulose, while synthetic polymers which can be used for producing composite polymer-based chitosan scaffold include polyglycolic acid (PGA), polylactic acid (PLA), polycaprolactone (PCL), polylactic-co-glycolic acid (PLGA), polyvinyl alcohol (PVA), polyacrylic acid, polyethylene glycol (PEG), and γ-glycidoxypropyltrimethoxy silane (GPTMS) (Lan Levengood and Zhang 2015; Pandey et al. 2017; Islam et al. 2020). The synthetic polymers are sometimes employed alone to make scaffold. They are also lower in cost and function better than natural polymers, but some of them show inadequate immune responses and lack of biocompatibility or toxicity, resulting in inflammation (Islam et al. 2020). Therefore, combining natural and synthetic polymers is purposed to obtain a nontoxic and compatible composite with better functional properties for tissue engineering applications.

A new development of chitosan-based materials for cartilage tissue engineering is constructed using dual network hydrogel of thiolated chitosan (CS-NAC) with silk fibroin (SF) via each gelation mechanism. The best formulated composition of CS-NAC/SF solutions produces hydrogels with better properties and supports the chondrocytes growth. These dual network gels are favorable in cartilage reconstruction (Liu et al. 2020).

A tripolymeric composite nanoparticle containing chitosan, poly-γ-glutamic acid, and pluronic is designed as a delivery device for wound healing and prepared by method of a simple ionic gelation. The composite shows stimulating regeneration of neocollagen and reconstruction of skin tissue (Lin et al. 2017).

Chitosan can also be employed for bone tissue engineering application. However, it is commonly hard to produce an ideal bone scaffold by the use of just chitosan or other single substance (polymer, inorganic or biological). The use of individual biomaterial has inadequate electrical performance, mechanical characteristics, and inconsistency quality for batch to batch production. On the other hand, inorganic materials are frequently fragile. The combining materials, including chitosan with two or more types of substances, have often been considered to show better properties of scaffolds. Materials investigated to be mixed with chitosan scaffolds include natural or synthetic polymers (collagen, alginate, silk fibroin, polyvinyl alcohol, polycaprolactone, etc.), biomaterials (hydroxyapatite, silicon dioxide, etc.), or pharmacological molecules (bisphosphonate, vascular endothelial growth factors, etc.) (Yao et al. 2016; Türk et al. 2018; Aguilar et al. 2019). A tissue

scaffolds for bone formation and tissue regeneration have been developed from chitosan/biphasic calcium phosphate (CS/BCP) functionalized with Arg-Gly-Asp (RGD) and bone morphogenetic protein-2 (BMP-2)-loaded nanoparticles. It is synthesized through the method of freeze-drying. The resulting scaffolds exhibit to stimulate cell adhesion and induce cell differentiation and are close to natural bone extracellular matrix (ECM) related to the composition and structural features. This study shows that the scaffolds are potential to be applied in bone tissue engineering (Gan et al. 2018). Other fabrications of artificial bone scaffolds are conducted to generate a superporous 3D collagen/functionalized multiwalled carbon nanotube/chitosan/hydroxyapatite (Col/fMWCNT/CS/HA) scaffold by the method of freezing and lyophilization. The resulting composite scaffold shows a significant enhancement of the biomineralization and mechanical characteristics compared to Col, CS, Col/fMWCNT, and Col/fMWCNT/CS scaffolds and is considered as potential for use in bone tissue engineering (Türk et al. 2018).

The application of chitosan for periodontal tissue engineering has been of interest since the last decade, although the research conducted is not as much as that of for bone tissue engineering. The beneficial properties related to its biocompatibility; bioactivity; antimicrobial, bioadhesive, and minimally immunogenic property; and ability to blend with other materials are to be considered as the reason of chitosan application for biodental or other biomedical purposes. Chitosan can be employed as a sole ingredient, and sometimes it presents better when blending with other compatible materials (Gu et al. 2019; Islam et al. 2020). Application of chitosan-based extrafibrillar demineralization strategy for periodontal treatment yields good results, including decreased collagen degradation initiated by endogenous proteases, keeping intrafibrillar minerals, inhibiting the growth of bacteria on dentin surfaces, and reducing water permeability of hybrid layers which then enhances the stability of resin-dentin bond (Gu et al. 2019).

3.4.2 Drug Delivery Systems

Many medications' efficacy is limited by their ability to reach the therapeutic action site. Only a small portion of the injected dose reaches the intended region, but the majority of the drug is disseminated throughout the body based on its physicochemical and biological properties (Sailaja et al. 2010). As a result, drug delivery systems have been one of the most active research fields for more than 50 years, with a focus on sustained/controlled and targeted pharmaceutical formulations capable of increasing patient compliance, personalizing, and enhancing therapeutic benefits. Drug delivery systems in a variety of forms, ranging from microparticles to nanoparticles, hydrogels to scaffolds, and membranes carrying pharmaceuticals embedded in organic, inorganic, polymeric matrices, or combinations thereof, are researched, some of which have already been marketed (Peptu et al. 2019).

Because of their higher bioadhesion to different tissues/mucosa, transfection properties, efflux pump inhibition, or permeation across biological barriers (nasal, oral, and vaginal mucosa or skin, gastrointestinal, ophthalmic, and blood-brain

barriers), chitosan/modified chitosan as cationic polymers provides increased bioavailability and therapeutic efficiency of various anionic drugs/genetic materials or hydrophobic/hydrophilic drugs (Peptu.et al. 2019). Due to its main amino groups, chitosan has a cationic property. Controlled drug release, mucoadhesion, in situ gelation, transfection, permeation enhancement, and efflux pump inhibitory characteristics are all due to these main amino groups. Most of these qualities can be improved further through chemical modifications (Bernkop-Schnürch, Dünnhaupt 2012).

Chitosan nanoparticles' physicochemical qualities can be changed to improve their use. The features of chitosan nanoparticles differ from those of chitosan, and they can be further improved for more effective drug delivery, particularly in terms of therapeutic efficacy. To increase intestine solubility, N-trimethyl chitosan chloride is manufactured. A nanoparticle formulation including thiolated chitosan is being developed to improve mucoadhesiveness. Chitosan opens tight connections across the membrane leading to an increase in this action by quaternization of chitosan, as seen in insulin permeation in Caco-2 cells. Increase of pH sensitivity can be achieved by grafting carboxylated chitosan with poly (methyl methacrylate). Physical modifications to chitosan can be accomplished by physically blending two or more polymers for particular purposes (Mohammed et al. 2017).

In situ gelling characteristics and mucoadhesive qualities cause chitosan nanoparticles to be employed in ophthalmic administration. Chitosan nanoparticles are used in oral drug delivery because they open mucous membranes' tight junctions and improve absorption. They are useful in the delivery of pulmonary drugs because of their positive charge. The permeability of various medicines can be increased with chitosan nanoparticles, thus enabling them to be delivered through the nose. Chitosan nanoparticles assist mucosal medication delivery by increasing the absorption of hydrophilic compounds. Chitosan nanoparticles are used as a vaccine adjuvant. These have been shown to be useful in the treatment of cancer. The physiochemical characteristics of chitosan nanoparticles have led to these uses. Biocompatibility, biodegradability, mucoadhesiveness, absorption augmentation, and in situ gelling are some of these properties (Garg et al. 2019).

For pharmaceutical purposes, chitosan must have a yellow or white appearance (flake or powder), a particle size of less than 30 m, a density of between 1.35 and 1.40 g/cm3, a pH of 6.5–7.5, a moisture content of less than 10%, a residue on ignition of less than 0.2 percent, a protein content of less than 0.3 percent, a degree of deacetylation of 70–100%, a viscosity of less than 5 cps, an insoluble matter of less than 1%, heavy metals (As) of less than 10 ppm, heavy metals (Pb) of less than 10 ppm, and no smell and taste (Sailaja et al. 2010).

The chitosan particulate system was made using a variety of approaches. The selected method is determined by parameters such as the required particle size, the chemical and thermal stability of the active components, the reproducibility of the kinetic profile of the final product release, and the residual toxicity of the final product. There are two methods for loading pharmaceuticals into the nanoparticle system, i.e., incorporation and incubation. Incorporation occurs during the nanoparticle creation process, whereas incubation occurs after the nanoparticles have been

formed. The drug is physically added to the matrix or adsorbed on the surface in each of these systems. By incorporating the drug during particle manufacture, it is possible to obtain maximum drug loading, and process parameters, such as manufacturing method, presence of additives, etc., affect the loading capability. Drugs that are both water-soluble and insoluble can be added to chitosan-based particle systems (Agnihotri et al. 2004). Hamman (2010) also mentions the usage of a chitosan-based polyelectrolyte complex as a viable carrier material in the drug delivery system.

The release of the drug from the particle system is affected by the degree of cross-linking, size, morphology, and density of the particulate system, and the treatment efficacy can be enhanced by the drug's physicochemical qualities and the presence of adjuvants. Meanwhile, the in vitro release was impacted by polarity, pH, and the presence of enzymes in the dissolving medium. Drug release from the chitosan particulate system is mediated by three mechanisms: (a) release from the surface of particle, (b) diffusion through the inflated rubbery matrix, and (c) release as a result of the erosion of polymer (Agnihotri et al. 2004).

Several chitosan nanoparticle applications as a drug delivery method in the pharmaceutical and biomedical areas have been studied extensively with varied treatment goals. Many researches on the use of chitosan nanoparticles as a delivery agent of drug for diverse therapeutic targets have been conducted. Chitosan nanoparticles can be employed to administer drugs parenterally, orally, or ocularly, as a vector for delivering nonviral gene and vaccine as well as for photodynamic treatment (Irianto and Muljanah 2011; Chakraborty et al. 2012; Rajalakshmi 2014).

3.4.2.1 Parenteral Administration

Nanoparticles can be delivered intravenously since blood capillaries have a diameter of roughly 4 m. The reticuloendothelial system in the liver, spleen, lungs, and bone marrow may quickly pick up particles with a diameter greater than 100 nm. Smaller particles, on the other hand, have a longer circulation duration. Positive or neutrally charged particles are removed more slowly than negatively charged particles (Rajalakshmi et al. 2014). Preparing hydrophilic nanoparticles with a charge of neutral surface is an effective way to decrease macrophage phagocytosis while also enhancing the success rate of loaded therapeutic particles treatment (Tiyaboonchai 2003).

Anticancer drugs are a promising field for research. According to Qi and Xu (2006), chitosan nanoparticles showed good anticancer efficacy when tested in vivo on multiple cancer cell lines. Agnihotri et al. (2004) describe the preparation of gadopentetic acid-charged chitosan nanoparticles for gadolinium neutron capture treatment. The nanoparticles' releasing qualities and ability to keep gadopentetic acid in tumor cells for an extended period of time imply that they can be used as intratumoral syringes in gadolinium neutron capture treatments.

According to Prabahara (2014), biodegradability, biocompatibility, excellent cell membrane penetration, high drug-carrying capacity, pH-dependent therapeutic delivery, multifunctionality, and long circulation period are the distinctive properties of chitosan-based nanoparticles that make them as one of the most promising

chemotherapy and cancer diagnostic delivery vehicles. Qi et al. (2005) show that chitosan nanoparticles efficiently suppress the growth of the human gastric carcinoma MGC803 cell line in vitro via a repeated mechanism and may be a useful drug in the treatment of cancer in humans.

3.4.2.2 Peroral Administration

Nanoparticles are being developed as oral delivery methods for macromolecules, proteins, and polynucleotides in order to protect labile medicines from enzymatic breakdown in the digestive tract. Chitosan nanoparticles transport anti-infective medicines such as antibacterial, antiviral, antifungal, and antiparasitic medications (Tiyaboonchai 2003; Chakraborty et al. 2012).

Qi et al. (2004) applied ionic gelation technique using tripolyphosphate anion to develop antibacterial chitosan nanoparticles which have antibacterial activity against *E. coli*, *S. choleraesuis*, *S. typhimurium*, and *S. aureus*. According to Loutfy et al. (2020), chitosan nanoparticles containing curcumin are utilized as an antiviral medication for the treatment of hepatitis C virus genotype 4a (HCV-4a). As a nanocomposite form, chitosan nanoparticles have the potential to synergize the antiviral action of curcumin. The nanocomposite exhibited a potent multitarget antiviral activity against HCV-4a entry and replication at the molecular and protein levels.

Following the report stating that blood sugar levels in diabetic rats were successfully lowered as a result of oral insulin nanoparticle therapy, this technique has been extensively explored (Tiyaboonchai 2003). Chitosan has been utilized as a carrier method to transport insulin molecules using polymeric nanoparticles. An in vivo study using chitosan/poly-(γ-glutamic acid) on a diabetic mouse model revealed that this nanoparticle system successfully reduced blood sugar levels. The combination of chitosan sulfate dextran nanoparticles was successful as a pH-sensitive delivery method, and insulin release was regulated by the polysaccharide dissociation mechanism (Sona 2010). Chitosan and poly-(γ-glutamic acid) nanoparticle systems were used for oral insulin administration.

Chitosan nanoparticle-based drugs have several advantages over traditional tablet or powder formulations, including the fact that they are nontoxic and compatible with soft tissue and cationic polysaccharides, adhere to the surfaces of mucosa, and momentarily open tight connections between neighboring epithelial cells. Because of their low stability, nanoparticles rapidly dissolve, allowing the loaded insulin to enter the systemic circulation via the opening paracellular route (Sung et al. 2012). Therefore, chitosan nanoparticles can enhance the absorption of insulin and interrelate with the intestinal mucosa (Alonso et al. 2005).

Zhang et al. (2010) present the ionotropic gelation technique for producing 100–400 nm nanoparticles for protein delivery from water-soluble chitosan with sodium tripolyphosphate utilizing bovine serum albumin as a drug model. Nanoparticles of water-soluble chitosan have been found to enhance and extend absorption of bovine serum albumin in the intestine. As a result, water-soluble chitosan nanoparticles might be used as a protein delivery method. Grenha et al. (2005) suggest using microencapsulated chitosan nanoparticles to transport protein

to the lungs, with chitosan increasing peptide absorption. Furthermore, chitosan nanoparticles have been proposed for the treatment of local lung disorders such as cystic fibrosis and cancer. To distribute hydrophilic peptides, Chen et al. (2007) produce a nanoparticle formulation using chitosan and sulfobutyl ether-7-β-sodium cyclodextrin.

3.4.2.3 Ocular Administration

Nanoparticles have been identified as possible ocular carriers for increasing medication bioavailability at the ocular surface. Nanoparticles of various kinds tend to attach to the eye's epithelial surface. The nanoparticles' extended residence time leads in a significantly slower elimination rate than traditional ophthalmological formulations, increasing the drug's bioavailability. Therefore, the delivery of active ingredients in eye drops such as allergy drugs, anti-inflammatory drugs, and beta-blockers is used as a direction for developing nanoparticles. Because of its ability to increase adsorption, chitosan is utilized as a carrier for eye drops (Tiyaboonchai 2003).

Salamanca et al. (2006) examined the potential for developing drug delivery methods for the ocular surface using nanoparticles of chitosan, which is an ionotropic gelation of chitosan with pentasodium tripolyphosphate in the manufacturing process. Chitosan nanoparticles indicated that they easily penetrated the conjunctival epithelial cells and had a good tolerance on the rabbit's eye surface. Silva et al. (2017) used mucoadhesive chitosan and hyaluronic acid for the development of eye drops for the effective treatment of eye disorders. The novel nanoparticle eye-drop formula's mucoadhesivity incorporates ceftazidime, antibiotics for treating eye infections. The eye-drop formulation contains 0.75% (w/v) polymer (hydroxypropyl) methyl cellulose in an isotonic solution made of nanoparticles consisting of sodium tripolyphosphate/chitosan-hyaluronic acid as a ceftazidime carrier.

3.4.2.4 Nonviral Gene Delivery Vector

The main idea of gene-based treatment is to cure pathological diseases by modifying the expression of disease-initiating genes at the cellular level by inserting new genes (DNA and RNA) into target cells. Therapeutic genes must overcome several physiological and molecular obstacles to reach target cells. Nonviral vectors provide a number of advantages, including a high degree of safety with a minimal immune response, ease of large-scale manufacturing, high efficiency for bioactive loading, and unlimited gene material size. In addition, compared to viral vectors, they have a reduced capacity to penetrate cells and a lower efficacy of gene transfection (Cao et al. 2019).

Chitosan has numerous potential benefits as a gene delivery tool, including high biocompatibility and cationic charge density as well as low immunogenicity and cytotoxicity (Lee et al. 2005; Shu and Zhu 2002). Furthermore, because of the presence of amine groups, chitosan is easily able to form a complex with negatively charged DNA. Naked nucleic acids are quickly destroyed by cell nucleases and are unable to get through the cell membrane. Simply combining positively charged

chitosan with negatively charged nucleic acid results in the production of polyelectrostatic complex at the nanoscale that protects an enzymatically degraded nucleic acid that has been condensed (Chang et al. 2010; Huang et al. 2005; Koping-Hoggard et al. 2001). Chitosan, which is positively charged, can form nanoparticle complexes when it interacts with nucleic acids having negative charge. Those vectors are released from the endosome (decomplexing process) into the cytoplasm after being taken in by endocytosis. Section 3.4.3 (Gene Therapy Systems) delves more into the use of chitosan as a vector for nonviral gene transfer.

3.4.2.5 Vaccine Delivery

Some characteristics of chitosan have been significantly enhanced after functional alteration, such as improved stability, mucoadhesivity, membrane permeability, and controlled release behavior, indicating that chitosan is a viable vaccine carrier system. To allow tailored vaccine administration, certain ligands, such as mannose, have been combined with a chitosan derivative for particular interactions with the target cell type (Xing et al. 2018). Meanwhile, nanoparticles have a strong adjuvant impact on vaccine parenteral delivery because they may be taken up directly by the cells that generate the antigen. Furthermore, nanoparticles are considered to have the ability to generate a mucosal protective immune response after oral and nasal delivery, which is one of the main aims of contemporary vaccinology. Peyer's patches are the major target for oral vaccination delivery. The vaccine is shielded from enzymatic breakdown on its journey to the mucosal tissues and is efficiently absorbed by M cells by integrating it into the nanoparticle system. In contrast to oral administration, nasal administration requires the vaccine to be delivered over a short distance, remain in the nasal cavity for 15 min, and not be subjected to degradative enzymes or low pH. Therefore, nasal vaccination delivery may not need nanoparticle formation and may instead be administered as a solution or powder, exceeding the formulation's contact time with nasal tissue (Tiyaboonchai 2003).

In a vaccine delivery method, Gordon et al. (2008) make chitosan nanoparticles that are loaded with the ovalbumin (OVA) protein antigen model. The amount of OVA that chitosan nanoparticles can adsorb is significant, with an adsorption efficiency of above 95%. In vitro studies utilizing immunologically activated murine dendritic cells reveal that chitosan nanoparticles release roughly half of the total protein over 10 days. Chitosan nanoparticles are used as an adjuvant in the administration of mucosal vaccinations by Mehrabi et al. (2018). The use of nanoparticles in the vaccine formulation improves antigen storage and immunogenicity, as well as targeted distribution and more precise release of the target agent. In the physicochemical characteristics of chitosan and vaccine delivery, the deacetylation degree and molecular weight of chitosan nanoparticles play a key impact.

3.4.2.6 Photodynamic Therapy

Photodynamic therapy (PDT) is becoming more widely acknowledged as a cancer treatment option. However, with a number of chemical treatments, photodynamic therapeutic agents such as photosensitizers (PS) have restricted applicability due to extended skin photosensitivity, poor water solubility, and insufficient selectivity.

Without interfering with their magnetic targeting, chitosan magnetic nanoparticles can provide excellent biodegradability, biocompatibility, water solubility, and nontoxicity. PS 2,7,12,18-tetramethyl-3,8-di(1-propoxyethyl)-13,17-bis-(3-hydroxypropyl) porphyrin (PHPP) is produced and developed as a medication delivery system and imaging agent using magnetic targeting chitosan nanoparticles (MTCNPs) (PHPP). PHPPMTCNPs have good targeting and imaging capabilities and may be utilized in PDT targeting monitored with magnetic resonance imaging (magnetic resonance imaging) (Sun et al. 2009). Rad et al. (2019) developed antimicrobial photodynamic therapy using chitosan nanoparticles doped indocyanine green, which reduces *Aggregatibacter actinomycetemcomitans* virulence factor and may be utilized as an addition to regular therapies for effective periodontal therapy in vivo.

Dental administration, colon drug delivery, liver drug delivery, kidney drug delivery, lung drug delivery, cancer-targeted drug delivery, and active targeting-receptor-mediated endocytosis (RME) are some of the other uses of chitosan for drug delivery that have been investigated (Rajalakshmi et al. 2014).

3.4.3 Gene Therapy Systems

Gene therapy is one of the most rapidly growing, promising, and expanding topics in modern medicine, with therapeutic promise for a wide range of hereditary disorders. The main idea behind this therapy is to correct errors in DNA produced by mutations in order to restore the functioning of the gene(s) in the cells. The procedure entails the delivery and introduction of new genes (DNA and RNA) into target cells capable of carrying the transgene's expression. Gene therapy has been widely used to treat a number of genetic disorders, including X-linked severe combined immunodeficiency (Hacein-Bey-Abina et al. 2003), cancers (Adair et al. 2012; Robbins et al. 2011), and infectious diseases (Hashiba et al. 2001; Ivacik et al. 2015; Bunnell and Morgan 1998), and disorders of the cardiovascular system (Stewart et al. 2006; Sundararaman et al. 2011; Vinge et al. 2008).

The quest for a viable vehicle for drug and gene delivery that possesses the entire set of characteristics necessary for therapeutic applications has recently received a lot of attention. During the early stages of development, gene transfer studies are focused on restoring gene function and control. Gene transfer is becoming an integral component of development for efficient therapy against many genetic illnesses, thanks to advancements in molecular biology techniques and a better knowledge of the genetic basis of disease. Direct distribution of bare therapeutic genes in vivo, on the other hand, is nearly impossible. The problems include gene vulnerability to nuclease breakdown in the plasma, nonspecificity targeted cells, and negative charge genes' difficulty to penetrate the negatively charged milieu of cellular membranes. Only six gene therapies have been approved, despite the fact that over 2500 have been finished or are ongoing clinical trials. Because of the difficulties described above, it is now widely accepted that naked gene transfer, either systemically or locally, is not physically feasible. As a result, developing

vectors of gene delivery systems that are safe and efficient in transferring therapeutic genes into target cells is the most challenging approach for human gene therapy. The viral and nonviral vector systems are the two types of gene transfer vectors that are widely employed.

Viruses have the capacity to efficiently transport their genes into host cells as infectious particles that are naturally evolved vectors. Because of these features, viruses are well-suited to the development of virus-based gene carriers. Virus vectors are now used in about 67 percent of gene therapy clinical studies. Adenovirus, retrovirus, vaccinia virus, adeno-associated virus, lentivirus, and herpes simplex virus are used by the majority of them (Giacca and Zacchigna 2012). The essential properties of viral genes include high levels of transfection effectiveness and fast transcription of the packaged gene, which are benefits of viral vectors in gene delivery systems. However, these benefits are frequently accompanied by significant drawbacks, limiting their clinical use. Unwanted immune responses, possible carcinogenic consequences, deadly inflammatory reactions, mutagenesis, poor target selectivity, and size limits on DNA cargo are among the most prevalent flaws of viral-based vectors (Bennett 2003; Check 2005; Hacein-Bey-Abina et al. 2003; Raper et al. 2003; Thomas et al. 2003).

Concerns about toxicity, immunogenicity, inflammatory potential, reduced carrying capacity for genetic material (the highest nucleic acid size is 34,000 bp, which makes gene editing difficult), viral vector cell selectivity, and complex industrial manufacturing have all been raised. The difficulties with viral vectors have accelerated the search for nonviral vectors as an alternative to viral vectors for gene delivery, such as delivery of gene silencing molecules (Needham et al. 2012; Pack et al. 2005).

Nonviral-based delivery systems, which were briefly discussed in Sect. 3.4.2.4, have a better safety profile because they are less immunogenic, don't have the risk of insertional mutagenesis, and can hold more nucleic acids. They are also more cost-effective and safe to manufacture. Attaching the required chemical sequences to the polymer can help increase the cell specificity of vectors. The abilities to form nanoparticles when combined with a nucleic acid, protect the nucleic acid from nuclease action, promote target cell endocytosis, escape from endosomes and lysosomes with ease, dispense the nucleic acid at the action site, and have low toxicity and a low cost of production are all characteristics of an ideal nonviral nucleic acid delivery agent.

Nonviral vectors may be divided into two groups, i.e., cationic lipids (liposomes and lipoplexes) and polymers (polyplexes). Cationic polymers have been extensively studied as gene delivery methods, with the most notable characteristics being their intrinsic ability to offer infinite gene packing capacities and the ability to undergo significant changes. Because of its high transfection effectiveness and robust gene complexation, polyethylenimine (PEI) is the most researched cationic polymer for gene delivery. However, due to a significant toxicity concern, its use in clinical studies is restricted. To solve the toxicity issue, chitosan-based carriers have gained popularity as a safe delivery mechanism for gene materials such as plasmid DNA (pDNA), oligonucleotide, and RNA. Compared to other cationic polymers,

chitosan is easy to form complex with negatively charged genes due to the presence of amine groups and a strong positive charge, as well as outstanding characteristics such as low toxicity, high compatibility, and low immunogenicity.

To increase the efficiency of the gene delivery method in practice, chitosan as a vehicle for carrying genetic material must be changed. To enhance the features of chitosan linked to gene transport, certain characteristics must be considered, such as molecular size, production of chitosan derivatives, degree of deacetylation, N/P ratio, and toxicity. The chitosan molecular weight is a key parameter that influences the size, stability, cellular absorption, and dissociation of the chitosan/DNA complex, impacting the polyplex's transfection effectiveness (MacLaughlin et al. 1998; Sato et al. 2001). Chitosan has a high water solubility in an acidic environment (pH 6.5) because its amino groups are protonated and become positively charged. Because of the hydrophobic effect of the chitosan backbone and the strong intermolecular hydrogen bonding generated by the –OH and $–NH_2$ groups, it becomes less soluble under neutral and alkaline pH settings. Reducing chitosan's molecular weight is the most effective way to enhance its water solubility in a neutral environment (Fu and Xiao 2017). By further hydrolyzing chitosan, smaller molecular weight derivatives of chitosan may be produced, which are thought to be more active than chitosan (Prasertsung et al. 2012). The usage of unmodified chitosan is limited due to its poor solubility under physiological circumstances, despite its attractive qualities as an efficient gene carrier. As a result, the development of chitosan derivatives with increased solubility for gene delivery has been thoroughly investigated and described in many publications (Chuan et al. 2019; Jiang et al. 2018; Layek and Singh 2017). The hydrophilic alteration of chitosan is reported as a covalent modification (Kritchenkov et al. 2017). The hydrophilic alteration of chitosan is reported as a covalent modification (Kritchenkov et al. 2017).

The positive charge and solubility of chitosan, gene binding ability to matrix, cellular absorption, and transfection effectiveness are all determined by the degree of deacetylation (DD) (Alameh et al. 2017; Yang et al. 2017). The more DD in a chitosan molecule, the more primary amines it contains. It entails a higher positive charge and a greater potential for gene complexation. According to one study, chitosan DD must be higher than 65 percent in order to form a stable combination with pDNA. Because siRNA has a strong negative charge, a higher DD (>80%) of chitosan is required to complex it and generate stable nanoparticles (Köping-Höggrd et al. 2001). Malmo et al. (2012) discovered that completely deacetylated chitosan was more effective than partially deacetylated chitosan in gene silencing, because completely deacetylated chitosan contained more amine groups (higher positive charge) and a greater decomplexing number in endosomal/lysosomal compartments.

The N/P ratio is the ratio of chitosan nitrogen (N) to gene phosphate (P). It calculates the net surface charge of polyplexes by combining the gene's molar stoichiometry with that of chitosan. The N/P ratio is a critical component that influences the stability of DNA complexes, their interaction with cells, and transfection effectiveness. A higher N/P ratio indicates more chitosan in the complex, which improves the polyplex's stability and, as a result, the contact with cells, resulting in

improved transfection efficiency. However, because the genetic material is bound too tightly to the matrix (too high stability), a complex with a very high N/P ratio may limit the capacity to release the gene from the complex, lowering transfection efficiency. An extremely low N/P ratio, on the other hand, will result in neutral or negatively charged complexes that will cluster owing to the lack of interparticle repulsive interactions, yielding poor transfection efficiency. At a nitrogen to phosphate ratio of 3:5, chitosan may effectively facilitate in vitro gene transfer. DNA-chitosan complexes with a positive surface charge of about +30 mV may be produced at this ratio with a size of 50 to 100 nm (Tiyaboonchai 2003). Various studies have found that in vivo observation using chitosan nanoparticles with a N/P ratio of >25 and a DD of 80–85% revealed a variety of issues, including blood incompatibility, poor gene release dosage, and nonspecific effects due to significant quantities of free excess cationic chitosan (Alameh et al. 2017; Malmo et al. 2012; Nielsen et al. 2010).

Bowman and Leong (2007) demonstrate the potential of chitosan nanoparticles as components with good gene delivery performance. Using the ionotropic gelation technique, Alonso et al. (2005) create chitosan nanoparticles carrying plasmid DNA. Nanoparticles have a high potential for macromolecular interaction and are a viable method for delivering pDNA transmucosally. In vitro, Kim et al. (2006) demonstrate that chitosan manosylate nanoparticles have high potential for gene delivery methods. Antiangiogenesis, apoptosis, and cell cycle induction are all activities of the chitosan manosylate/plasmic complex encoding murine IL-12, which can suppress tumor development. Lu et al. (2011) produce hybrid hyaluronic acid (HA)/chitosan (CS) nanoparticles that can deliver exogenous genes into primary chondrocytes for the treatment of osteoarthritis.

Zhou et al. (2018) show that a nonviral gene delivery method based on hyaluronic acid-chitosan (HA/CS) may be used to produce the cytokine response modifier (CrmA) gene. This system offers 3 weeks of plasmid DNA release and aids in increasing CrmA levels to high levels in a short length of time. In a mouse model of osteoarthritis, suppression of interleukin (IL)-1 in synovial tissue by HA/CS-CrmA nanoparticles reduces cartilage degradation and synovial inflammation. By suppressing IL-1 production in the synovium, HA/CS-CrmA nanoparticles may be controlled to give local distribution in the joint area, preventing osteoarthritis development.

3.4.4 Antimicrobial Material

Chitosan has been known as biomaterial with antimicrobial activity against large number of microorganisms including bacteria (Gram-positive and Gram-negative bacteria), yeast, fungi, and also microalgae that are potential for the use in biomedical, cosmetics, food, and agricultural fields. Application of chitosan for antimicrobial can be presented as original or its derivatives form (COS, N-acyl chitosan, quaternized chitosan, etc.) in various physical form of solution, films, beads, fibers, membranes, hydrogel, and composites (Atay 2019). The antimicrobial activities are

related to the intrinsic (molecular weight, degree of deacetylation, concentration, etc.) and extrinsic factors such as pH, temperature, reactive time, and type of microorganisms (Divya et al. 2017; Shih et al. 2019). However, the antimicrobial activities of COS are not determined by their molecular weights, but depend on the type of the microorganism tested (El-Sayed et al. 2017).

Mechanism of antimicrobial activity of chitosan has not been well understood. However, there are several hypotheses related to the mechanism of chitosan antimicrobial action which can be classified based on nature and function of chitosan, as follows:

- Chitosan's polycationic nature: The antimicrobial action is due to the interaction between amino group in chitosan with positive charge and surface bacterial molecules with negative charge causing release of intracellular constituents; the binding makes the charge of bacterial surface to be reverse or neutral (Jeon et al. 2014; Kravanja et al. 2019).
- Binding of chitosan to DNA's bacteria (mRNA inhibition): The binding causes inhibition of mRNA and therefore synthesis of protein; the low molecular weight chitosan ($\leq$ 50 kDa) and nanochitosan may get through the cell wall of bacteria and inhibit the transcription of DNA (Divya et al. 2017; Kravanja et al. 2019).
- Agent of chelation (nutrients and certain important metals): Chitosan binds certain metals and hence inhibits the growth of microbes and toxin production. Antimicrobial activity of chitosan is occurred in an acidic medium (<6.5) when it is as a cationic polymer (Divya et al. 2017; Kravanja et al. 2019; Shih et al. 2019).
- Blocking agent: Chitosan layer covers the outer membrane of bacteria and avoids bacterial cells from obtaining nutrient from the environment. It also inhibits the growth of aerobic bacteria by blocking the oxygen pathway (Kravanja et al. 2019).

Chitosan with large amount of amino groups is potential for antimicrobial agent. The increase of free amino groups enhances the antimicrobial activity. Thus, chitosan with higher degree of deacetylation exhibits a higher activity compared to chitosan molecule with a lower DD. The inhibitory effect of chitosan varies between Gram-positive and Gram-negative bacteria. The mechanism of activity is relatively complicated. Gram-negative bacteria have hydrophilic outer membranes with lipopolysaccharide content to protect the bacteria from the environment and filter out hydrophobic toxins. Gram-positive bacteria have a thick layer of peptidoglycan and teichoic acid which function as outer membranes and act as scaffolds for membrane-bound enzymes which are essential for cell wall growth and degradation (Swoboda et al. 2010; Shih et al. 2019).

Antibacterial effect of chitosan is investigated for both Gram-negative and Gram-positive pathogenic bacteria, such as *Salmonella paratyphi* and *Staphylococcus aureus*. It is predicted that chitosan positively charged interacts with negatively charged components of bacterial cell walls and membranes disrupting metabolism of normal cell (Islam et al. 1970; Escárega-Galaz et al. 2017). Many studies also exhibit that antimicrobial effect of chitosan targets cell surface, indicating that

chitosan is a good option for antifungal therapy (Shih et al. 2019). The activity of chitosan against pathogenic fungus has been investigated either in the form of solution, film, gel, or composite of natural chitosan, nanochitosan, COS, or chitosan derivatives, including the fungi *Aspergillus flavus* (de Oliveira Pedro et al. 2013; Fawzya et al. 2019), *Trichophyton rubrum* (Mei et al. 2015), and *Candida albicans* (Peña et al. 2013; Pu et al. 2014; Shih et al. 2019).

3.5 Conclusion

Chitin, chitosan, and their derivatives, particularly COS, have been explored by scientists from countries all around the world to reveal the various benefits of their uses, especially for medicinal purposes. Modification of chitosan chemically, physically, and enzymatically has produced derivatives with better physical, chemical, and pharmaceutical properties so that it can expand their uses. Thus, the emergence of more complex types of diseases from time to time must be balanced with the development of more advanced medical and pharmaceutical sciences in order to produce drugs or medicines for therapeutic purposes that are more potent, more targeted, and faster in healing. Based on the existing studies, chitosan has demonstrated an opportunity to contribute to drug and medicinal developments.

It is estimated that the global demand for chitin and chitosan to support the production of several types of drugs for various diseases in the future will increase in line with the projection that the demand for chitosan in the global market will also increase as previously discussed. Chitosan and COS are mostly processed from the shells of crustacean including crabs, lobsters, crayfish, shrimps, krill, woodlice, and barnacles. The market of crustacean can be segmented into shrimps, prawns, lobsters, crabs, and others. The total catch of shrimp, lobster, and crab shows an increasing trend and is estimated at more than 800 thousand tons in 2016, while the total production of crustaceans from aquaculture is around 7862 thousand tons in 2016 (FAO 2018). The crustacean processing industry is generally scattered, so the collection of crustacean shells is a challenge that must be handled properly for the sustainability of the chitosan processing industry. Meanwhile, the available source of crustacean shells as raw material must be ensured to meet the quantity and quality requirements for sustainable chitosan production. In addition, chitin and chitosan production technologies that are more environmentally friendly must be continuously developed, especially in relation to the use of chemicals during processing.

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Marine Collagen for Delivery of Therapeutics

4

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Abstract

Collagen is the most plentiful protein in mammals and considered the most significant part of the body structurally and functionally. The marine sponge is also an important and unexplored source for collagen productions until now. Among the biopolymers, collagen signifies one of the most utilized biomaterials in view of its superb biocompatibility, biodegradability, and low antigenicity, confirmed structure, and biologic features. The increasing enthusiasm for the utilization of marine collagen biomaterials and their important properties makes them useful for various biomedical applications. This chapter centers on the growing interest in marine collagen (MC)-based platforms for therapeutic applications. Specific consideration is given to the bioactive properties of MC that are being investigated in preclinical and clinical examinations. In this chapter, an analysis of the work reported on collagen derived from marine species and fish trashes is presented. This chapter also provides structure, extraction, sources, and various therapeutic applications about MC obtained from marine sources. The MC market is quickly examined to feature the area of interest and the most productive areas of interest. The present status and upcoming possibilities of

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marine collagen-based biomaterials are examined in general along with appropriate examples drawn from existing literature.

Keywords

Marine collagen · Therapeutic · Anti-HIV: pharmaceutical · Marine collagen peptides · Obesity · Drug delivery

4.1 Introduction

Collagen molecules signify the most abundant construction material in the human body, where they give mechanical stability, flexibility, and strength to the organism, for example, tendons, ligaments, bone, and the extracellular matrix (ECM) (Gautieri et al. 2011; Fratzl 2008; Buehler and Yung 2009; Rainey et al. 2002; Fraser et al. 1983; Hulmes et al. 1995; Orgel et al. 2001; Currey 2002; Orgel et al. 2006; Aladin et al. 2010). These are also called smart materials because they change their behavior in response to mechanical force by modifying their structures from the subatomic level (Chang and Buehler 2014; Wang 2006; Magnusson et al. 2010; Kjær 2004). It is realized that almost all collagen-based tissues are composed of progressive structures, where the lowest hierarchical level consists of triple-helical collagen molecules (Fig. 4.1) (Gautieri et al. 2011; Chang and Buehler 2014). Collagen is a trimeric particle composed of three chains of polypeptide twisted into a triple helix that frames homotrimers or heterotrimers, subject to the kind of collagen (Lee et al. 2001; Cen et al. 2008). High biocompatibility, biodegradability, and bio-renewal as well as low antigenicity have been found in collagen (Lee et al. 2001; Sayani and Ronald 2014; Carvalho et al. 2018; Asghari et al. 2017; Albu et al. 2011). The communication among collagens and cells is attained by cell surface receptors (Vogel 2001; Hartford Svoboda and Reenstra 2002). These features have contributed to the improvement of a significant range of biomedical devices based on collagen, including drug delivery systems, hemostatic specialists, tissue engineering, etc. (Lee et al. 2001; Cen et al. 2008; Sayani and Ronald 2014; Carvalho et al. 2018; Asghari et al. 2017; Albu et al. 2011; Karsdal 2016). Likewise, collagen has organized structure and arrangement of the amino acid organization among species, contributing to the referenced properties when taking into account the use of animal collagen in the biomedical perspective. There are 28 distinct collagen forms called in sequential order of disclosure with Roman numeral assignments (I–XXVIII) (Karsdal 2016). Collagens are commonly comprised of three long amino acid chain helicoidally molded. Bindings of proline and hydroxyproline at the Y position are the base structure of the chains of two other amino acids and repeat grouping $(Gly\text{-}X\text{-}Y)_{n.}$ The chains are classified into main, secondary, and tertiary structures with a final fibril structure (Ferreira et al. 2012). The various kinds of collagen are ordered relying upon domain structure and their superstructural group. Every collagen has a particular α-chain with its own space structure which adds to the characterization by collagen type. Collagens can be fibril-forming, fibril-related with interrupted triplex helix such as collagen types, fibril-related with interrupted

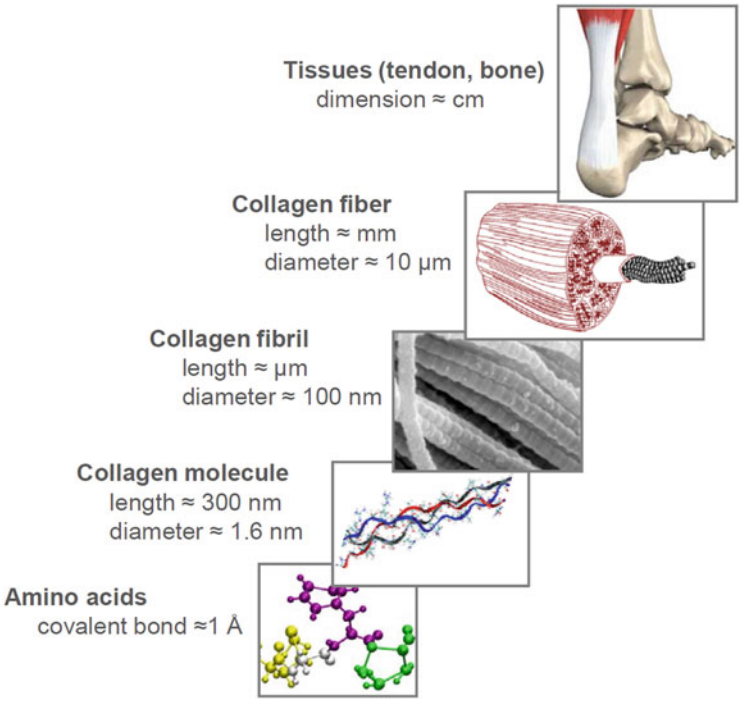

Fig. 4.1 Hierarchical structure of collagen protein materials. Reprinted with permission from Ref. (Gautieri et al. 2011). Copyright (2011) American Chemical Society

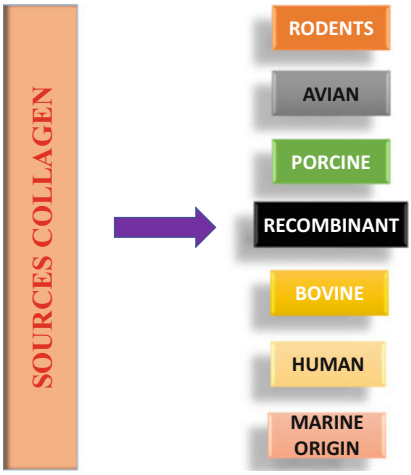

Fig. 4.2 Popular sources of collagen

triple helix-like, and network-forming (Coppola et al. 2020). Collagens in each class have their own specific capacity and add to higher degree tissue structures. Figure 4.2 signifies the most well-known sources of collagen (Tharindu et al. 2020). It is considered as the primary basic protein of connective tissues (Shoulders and Raines

2009). It signifies around one-fourth of the complete protein content in many creatures (Prockop and Kivirikko 1995). It is a fibrous protein framed by three polypeptide chains, organized as a triple helix enclosed around one another (Ramshaw et al. 1998). This chapter focuses on the comprehensive description of marine sources, extraction, and various applications of marine collagen in the biomedical field, particularly for therapeutic applications.

4.2 Marine Collagen (MC)

More than 70% of the Earth is occupied by oceans that are populated by a range of life forms. A fascinating source of collagen and bioactive substances used in many fields, as well as the medicinal, cosmetic, and food industry, is the MC. MC has attracted a wide reasonable and industrial interest on the account of its solubility in water, safety, biocompatibility, high biodegradability, ease of extraction which converts into high performance, and low production costs. MC can be categorized into two: collagen derived from invertebrate species, including fish (Liu et al. 2014), squid (Ando et al. 2001), octopuses (Nagai et al. 2002), wipes (Lin et al. 2011), and marine sponge (Silva et al. 2016), which are marine collagen sources, and collagen from marine vertebrates, incorporating fish and marine mammals, which is another class of marine collagen. Type I collagen is mainly derived from marine sources, much of use as essential biomaterials for the development of scaffolding, through various collagen types and can be attained correspondingly from fish cartilage, jellyfish, and marine sponges (Coppola et al. 2020; Tharindu et al. 2020; Shoulders and Raines 2009). Type I collagen is the protein most commonly isolated from marine organisms, as it is a significant biomaterial in the tissue designing field. In recent times, collagen derived from marine sources has been widely explored (Fig. 4.3) (Tharindu et al. 2020). The various important characteristics of marine collagen are displayed in Fig. 4.4. If the fish ligament is the primary source, collagen type II may be achieved. Various methods for evaluating the antioxidant effects of peptides obtained from fish have also been used, together with radical and hydroxyl scavenging tests (Jeevithan et al. 2015). Antimicrobial peptides have cationic residues that facilitate their interaction with microbial germ membranes (Bardan et al. 2004). Antimicrobial peptides in marine species, however, contain new antibiotics forms. Papain, pepsin, trypsin, alkaline proteases, acid proteases, aromatic proteases, and so on were enzymes used to isolate antimicrobial fish peptides and bacteria vulnerable to these antimicrobial peptides. Some jellyfish, such as the strip jellyfish (*Chrysaora* sp.), are rich in collagen type II (Barzideh et al. 2014), and marine sponges such as *Chondrosia reniformis* are identified to have type IV collagen (Pozzolini et al. 2012). These are the common sources of MC and found tremendous applications for medical and industrial use (Lin et al. 2011). Table 4.1 sums up various categories of collagen acquired from marine sources. Collagen of marine origin has different characteristics from those obtained from terrestrial sources. It has been found to have diverse amino acid composition, glycosaminoglycan content, and denaturation temperature (Bardan et al. 2004). These different characteristics, added to the minimal possibility of transmission of diseases

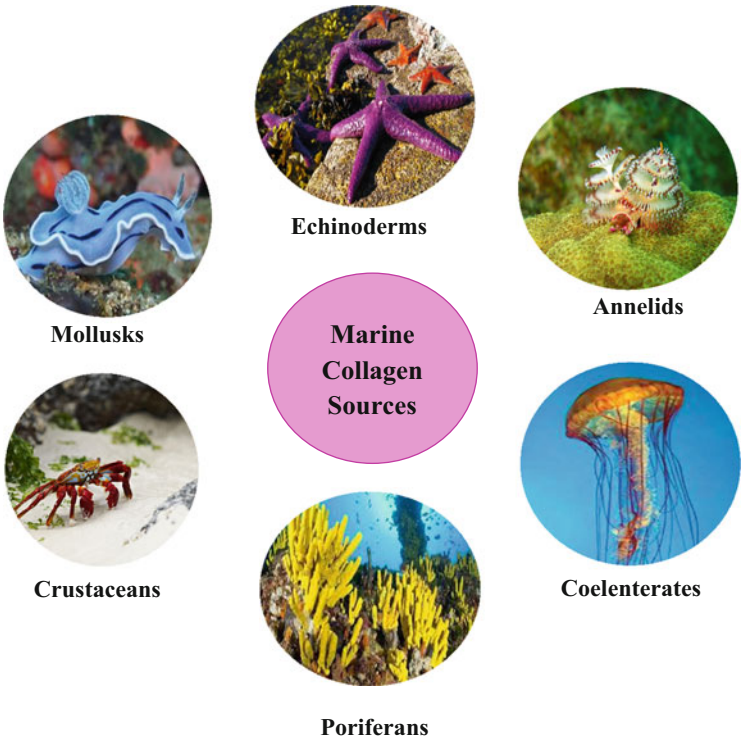

Fig. 4.3 Marine sources of collagen

communicable to humans and of not having religious restrictions, make marine collagen superior to its terrestrial equivalents. However, a lot of marine biodiversity has not yet been explored from this point of view (Pal and Suresh 2016).

4.2.1 Marine Collagen Peptides

A perfect source of bioactive proteins and peptides is marine collagen, which has much of the global biodiversity (Nikoo et al. 2011). Generally, bioactive peptides are acquired from entire protein molecules through enzymatic hydrolysis and fermentation. Collagen may be hydrolyzed into collagen hydrolysates or gelatin hydrolysates under desirable conditions in its denatured (gelatin) or undenatured type. In this process, collagen is mixed with proteins such as papain, pepsin, E-erase prop, and trypsin to form short peptide chains with bioactive properties (Fan et al. 2013). Enzymatic hydrolysis is one of the most widely recognized techniques utilized for the formation of bioactive peptides. Enzymatic hydrolysis was performed by utilizing an autolytic procedure or commercial protease (Nikoo et al. 2014). The enzymatically hydrolyzed oligopeptide compounds in collagen, cartilage, and

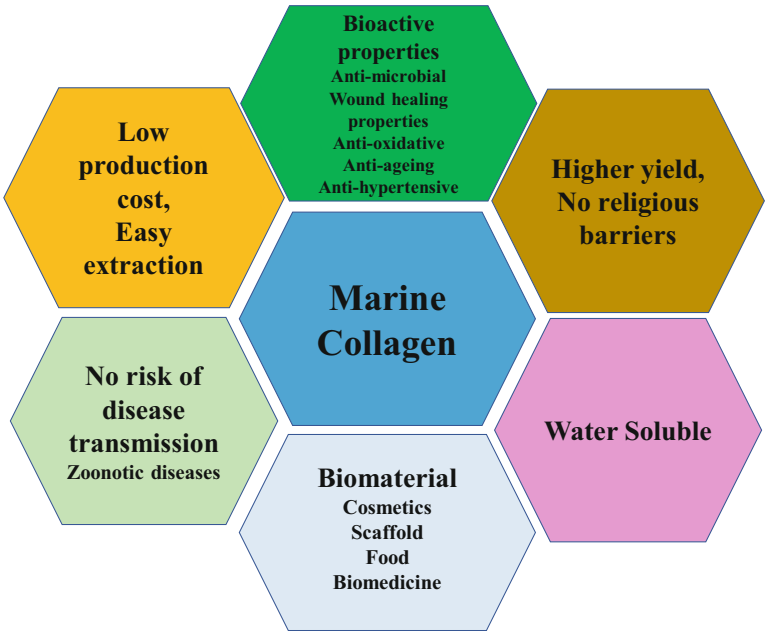

Fig. 4.4 Important properties of marine collagen

Table 4.1 Marine species for the extraction of collagen

Collagen type	Type	Collagen sources
I	Fibril-forming collagens	Jellyfish (Cheng et al. 2017), sea urchin (Benedetto et al. 2014), Persian Gulf squid skin (Delphi et al. 2016), crown-of-thorns starfish (Tan et al. 2013), codfish skins (Alves et al. 2017), salmon skins (Raftery et al. 2016), fish scales of oil sardines (Muthumari et al. 2016), minke whale (Nagai et al. 2008), catfish (Bama et al. 2010), yellowfin tuna skin (Woo et al. 2008), chum salmons (Wang et al. 2010a)
II	Fibril-forming (fibrillar)	Ribbon jellyfish (Barzideh et al. 2014), silvertip shark cartilage (Jeevithan et al. 2014)
III	Fibril-forming (fibrillar)	
IV	Basement membrane collagens	Sponges (Pozzolini et al. 2012), marine sponge (Lin et al. 2011)
V	Fibril-forming collagens	Jellyfish medusa (Calejo et al. 2012)

marine fish bones are marine collagen peptides (MCPs), rich in glycine, glutamic acid, proline, and hydroxyproline (Ri et al. 2007). Furthermore, in in vitro studies, it has been discovered that fish collagen peptides have a high level of antioxidant

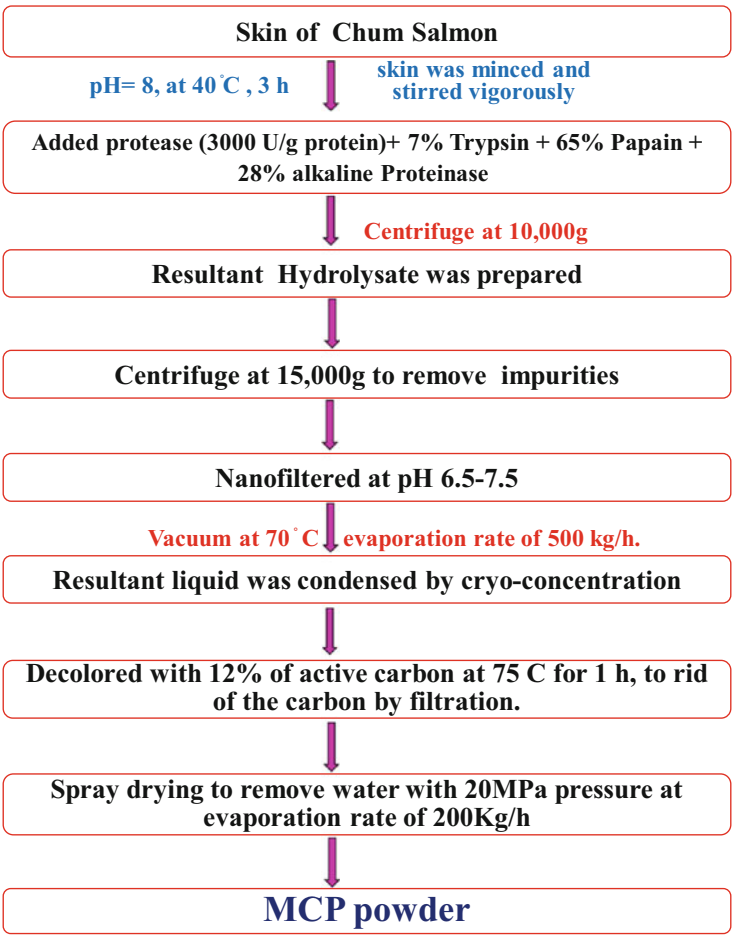

Fig. 4.5 Schematic representation of extraction of marine collagen peptides (MCPs) from chum salmon (*Oncorhynchus keta*) skin

activity to remove free radicals and inhibit lipid peroxidation (Zhuang et al. 2009), whereas in another study, MCPs were recommended to show substantial radical scavenging activity and antagonistic to lipid peroxidation action tested with a 2,2-diphenyl-1-picrylhydrazyl radical scavenging assay and linoleic acid peroxidation process, correspondingly (Zhentao et al. 2009). Schematic representation of MCP extraction from chum salmon skin is demonstrated in Fig. 4.5 (Pei et al. 2010).

4.3 Collagen Extraction

The higher number of distributions about marine collagen discovers a developing enthusiasm from the pedagogic system to think of new gainful sources. Marine collagen can be gained from various sources, and the emphasis is the extraction from the abundant ones with strategies that could be effectively scaled up so that processes can be beneficial for industrial applications. Therefore, with the right technology, marine collagen can be used as a potential alternative to using mammals for this purpose. From the marine environment, different strategies for the acquisition of collagen have been suggested, based on marine sources. An overall method for the separation of collagen from fish and marine sources is by taking into consideration three stages: synthesis, extraction, and recovery (Nagai et al. 2010). The procedure includes washing, the partition of animal chunks, and reducing the size of the sample by chopping or shredding them. To allow the resulting chemical pre-treatment activities to eliminate non-collagenous proteins, stains, or fats, reducing the size of these samples is important. The standard technique uses an essential sodium hydroxide pre-treatment, which does not allow structural alteration of the chains of collagen, alcohol, and oxygen peroxide separate from non-collagenous protein, fats, and color, during the expulsion phase (Zhang et al. 2009). In addition, the use of NaCl as a substitute for NaOH was also suggested for the abstraction of non-collagenous proteins from the skin of codfish (Sadowska et al. 2003). Moreover, EDTA is recommended for demineralization purposes to improve collagen production from hair, ligament, and scales (Kittiphattanabawon et al. 2005). On the other hand, HCl can likewise be utilized (Żelechowska et al. 2010). Collagen extraction from the umbrella's species results after the removal of the inner and external skin with a surgical blade. The tissue was lyophilized and frozen at −80 °C before use. In short, the freeze-dried umbrellas were treated with 0.1 M sodium hydroxide to eliminate non-collagen proteins. When collagen extraction takes place only with acid, the resulting material is called acid-soluble collagen (ASC). Acetic acid is often employed, although citric acid and lactic acid are also used in collagen production from marine animal tissues (Nagai et al. 2000). Yusoff et al. (2013) proposed a different approach to separate collagens from amphibian species that blends acidic preparation with physical and mechanical preparations, including pH enhancement, uniformity, mixing, and sonication. The extraction yield increases dramatically compared with the methods of standard extraction by extending physical procedures in jellyfish (Khong et al. 2018). The derived collagen is called pepsin-soluble collagen (PSC) at the stage where the protein pepsin is used in the extraction process. This procedure is very beneficial because proteases cut the telopeptide cross-linked regions without interrupting the triple helix integrity, thus hydrolyzing some non-collagen proteins and increasing the collagen purity (Zhang et al. 2007). Catalyst is also used to acquire explicit protein material, high outputs, and decreased waste and to reduce antigenicity due to telopeptides (Schmidt et al. 2016). But when a large measurement of pepsin is employed for a higher time period, PSC output may decrease for the reason that the collagen can decompose, undermining the integrity

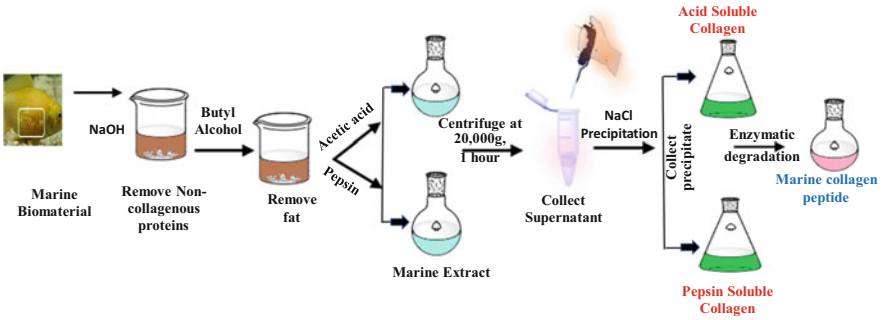

Fig. 4.6 A flowchart for extracting acid-soluble collagen and pepsin-soluble collagen from the skin of the marine fish

of the triple helix structure (Jongjareonrak et al. 2005). The detailed extraction procedure of ASC and PSC is displayed in Fig. 4.6 (Jongjareonrak et al. 2005).

For the most part, collagen is precipitated during the recovery period by applying NaCl to the final concentration, i.e., 2.3–2.6 M. The subsequent precipitate, dissolved in 0.5 M acetic acid, dialyzed, and lyophilized, is collected by centrifugation (Silva et al. 2014). In general, collagen extraction from jellyfish is based on solubilization in a solution of 0.5 M acetic acid, followed by dialysis salting against a solution of Na_2HPO_4. By centrifugation, collagen precipitates were separated, solubilized into acetic acid, and reprecipitated for purification with 0.9 M NaCl. ASC can likewise be treated with pepsin to produce atelocollagen (Song et al. 2006). Zhang et al. (2007) compared the properties and composition of collagen extracted from grass carp with the calfskin. The grass carp skin was used to isolate the collagen with a yield of 46.6% by the PSC method. The grass carp skin collagen contained low imino acid content as compared to mammalian's collagens. Several peptide maps of collagens of grass carp skin and calfskin collagens showed that there might be several variations in sequences or conformation of amino acids. Therefore, grass carp will be used for industrial applications as an alternative to calfskin and pigskin collagen (Zhang et al. 2007). Many peptide maps of grass carp skin collagens and calfskin collagens showed that several differences in amino acid sequences or confirmation could occur.

4.4 Therapeutic Effect of Marine Collagen

The utilization of collagen-based materials in the administration of drugs implies the investigation of multiple viewpoints, viz., in vivo toxicity, bioavailability, solubility, and body tissue captivation with specific target administration. The significant concern has been raised with respect to the protection and medical credibility of the administration of marine collagen peptides in light of the fact that MCPs from various cause can trigger the innate immune response through the initiation and overproduction of responsive oxygen species (ROS) by a Toll-like receptor

4 (TLR4)-intervened NADPH oxidase (NOX4) (De Luca et al. 2016a). Various studies on MC, collagen-derived peptides, and gelatin from natural sources were carried out to find future medicinal and pharmaceutical applications, as well as gelatin from natural sources.

4.4.1 Drug Delivery

Currently, the area of drug delivery from various biopolymer/biomaterials has an expanded improvement because of its advantages contrasted with the fundamental administration. Among the biopolymers, collagen is one of the most utilized, being an appropriate biodegradable polymer help for drug delivery systems, providing the benefit of natural biopolymers with hemostatic, vitreous implants and wound dressings properties (Lee et al. 2001; Albu et al. 2010; Arpornmaeklong et al. 2008; Davidenko et al. 2010; Goissis and De Sousa 2009; Gonçalves-Neto et al. 2002; Gotterbarm et al. 2006). The use of polymers for the film covering of solid pharmaceutical formulations has grown exponentially. Several investigations have demonstrated that it is workable for a limited quantity of medication to either break down in the covering solution during the film development or relocate from the tablet center to the surface of the film (Dansereau et al. 1993). In addition, collagen fibrils and their denatured derivatives, such as gelatin, are the primary structural and functional components in different biomedical applications (Coppola et al. 2020; León-López et al. 2019; Meyer 2019; Gorgieva and Kokol 2011; Lu and Guo 2018; Sorushanova et al. 2019). For the most part, cross-linking agents such as glutaraldehyde or the approval of cross-linking by UV or g-radiations reduces the in vivo absorption of collagen. Furthermore, collagen, along with gene therapy nanoparticles, base matrices for cell culture systems, and healthy blood vessels and artificial valves, can be used as regulatory material for transdermal release. Several pieces of research have been undertaken from numerous sources to manufacture collagen, including skin and fish bones from the seafood manufacturing industry. Consequently, Veeruraj and his collaborators (Veeruraj et al. 2012) conducted studies to understand the drug-releasing behavior of the implantable type I collagen derived from the waste from the external eelophish skin (Evenchelys Macura) as a drug carrier against human pathogenic microorganisms. ASC and PSC gels were successfully distributed with standard pharmaceutical medicines like ampicillin and tetracycline and isolated collagen films. The drug's antibiotic efficacy (ampicillin and tetracycline) has been reviewed by the process of agar disc diffusion in which the zone of inhibition was formed by them. This method has confirmed to be employed for drug delivery in the future (Veeruraj et al. 2012). Due to the beneficial properties of marine sponge collagen such as adsorption ability, nonantigenic nature, non-toxicity, and biocompatibility, it can be used as a drug delivery tool (Chak et al. 2013). Microparticles derived from collagen of marine sponges were used for the cutaneous administration of all-trans-retinol by Swatschek et al. Retinol-loaded sponge collagen was studied in the skin of hairless mice and retinol penetration into the skin was greatly improved (Swatschek et al. 2002). As

penetration stimulators for the transdermal administration of 17β-estradiol-hemihydrate in hormone replacement therapy, *Chondrosia reniformis* spongy collagen nanoparticles were developed by Nicklas et al. (2009a). These collagen nanoparticles were synthesized by conventional alkaline hydrolysis processes and were later distinguished by various spectroscopic techniques. Compared with a commercial gel, the collagen nanoparticle hydrogel loaded with estradiol permitted a prolonged release of estradiol and produced a much-improved estradiol absorption. Sponge collagen nanoparticles are also encouraging materials for the delivery of transdermal drugs (Farstvedt et al. 2004). Collagen-based films seem to be encouraging from this point of view based on the fact that their three-dimensional porosity/structure can be altered as desired. Marine sponge collagen (Porifera, Dictyoceratida) has been described and processed into polymer films that contain l-cysteine hydrochloride for wound healing purposes (Pozzolini et al. 2012). A large number of topical wound dressings are now accessible, such as films, foams, antiseptic-containing hydrogels, antibiotics, and/or healing factors (Langasco et al. 2017). In vitro experiments have shown that cysteine is released more steadily than the pure substance at the injury site. This makes the system interesting and able to provide bio-based collagen, which can be used as a biocompatible transport system as it adds the known healing properties of cysteine with the beneficial properties of the collagen/proteoglycan network (Panduranga Rao 1996). The progress in the use of collagen as suitable biomaterials and in drug delivery devices is reviewed by various authors (Lee et al. 2001; Panduranga Rao 1996; Chvapil et al. 1973; Khan and Khan 2013). Gil et al. (2016) utilized reused collagen films for controlled drug delivery devices. They concluded that examined films have a great ability for drug delivery and extraordinary efficacy in hindering microorganism's expansion. Use of collagen-model triple-helical peptide amphiphiles (PA) for CD44-targeted drug delivery devices was reported by Ndinguri et al. (2012). They recommend that PA targeted liposomes may signify another class of nanotechnology-based drug delivery devices. The sustained release of studies for antibiotics, for example, gentamicin, utilizing collagen as a drug delivery carrier was investigated by Wahlig and Dingeldein (1980). Fujioka et al. (1995) reported a novel drug delivery system for proteins utilizing collagen mini pellets as a carrier material. This system is appropriate to different sorts of biologically active proteins and likely to encourage their use in biomedical applications. Protein nanoparticles as suitable materials to be employed for various pharmaceutical devices were reviewed by Jain et al. (2018). The use of new drug delivery schemes to focus on drugs for explicit parts of the body could be an option that can address these basic problems. The enteric coating of granules, tablets, and cases has been used as often as possible for decades, prevents the processing of oral medications in the stomach, and guides the sustained release of the active substance into the upper digestive system. Moreover, the collagen found in *Chondrosia reniformis* marine wipe is stable against pepsin and trypsin and insoluble in acid media (Imhoff and Garrone 1983).

4.4.2 Metabolic Syndrome

Marine collagen peptides (MCPs) were shown to have positive consequences for metabolic syndrome, which involves type 2 diabetes, abdominal obesity, hyperlipidemia, and hypertension, among other unhealthy diseases (Alberti et al. 2005). Angiotensin I conversion enzyme (ACE) may be inhibited by marine collagen peptides (Kim et al. 2012) and so can probably diminish hypertension and hyperlipidemia (Wang et al. 2010b). Furthermore, MCPs indicate an increased response to insulin and decrease the risk of related metabolic complications (Zhu et al. 2010a) in people who have resistance to insulin. Similarly, MCPs demonstrated greater glucose and lipid metabolism and blood pressure in patients with type 2 diabetes (Cui-Feng et al. 2010), though the mechanisms behind the effects of MCPs are not completely understood. A significant factor in the pathophysiology of type 2 diabetes is insulin obstruction. Oxidative stress increased inflammation, and increased peroxisome activity was involved in insulin-resistance pathogenesis, including proliferating-activated receptor and type 4 glucose transporter (GLUT4) (Tangvarasittichai et al. 2016). In patients with type 2 diabetes, MCPs extracted from wild salmon skin are likely to decrease insulin resistance. MCPs have also been thought to have a positive impact on the reduction of oxidative stress, inflammation, and GLUT4 and PPAR-a expression (Zhu et al. 2017a). MCP therapy has been shown in these patients to aid in treating hyperglycemia and high blood pressure. MCP therapy was also beneficial to manage hypertension as revealed by a fundamental reduction in SBP, DBP, PPD, and MAP levels, compares and the baseline of these patients and the treatment with MCP indicates substantially decreased levels of diabetes-related renal impairment and hypertension as seen in these patients, CR levels are drastically decreased. These therapeutic results indicated that MCPs could be useful for enhancing the metabolism of insulin-sensitive glucose, renal function, and control of the blood pressure in patients, inflammation, and positive modulation of the development of bradykinin and adiponectin. These findings, together with relative protection, show that MCPs can work as an interventional supplement in patients with both T2DM and hypertension (Zhu et al. 2010b; Wang et al. 2008). MCPs have been recognized to substantially decrease the sugar level in the blood, total triglycerides, total cholesterol, low-density lipoprotein, and free fatty acids. However, it has improved insulin sensitivity, high-density lipoprotein (HDL), and adiponectin levels (Zhu et al. 2010c). Diabetic patients have been recognized to have overall increased plasma CF6 levels, which are positively correlated with blood lipid and glucose levels (Li et al. 2007). The medium and high doses of MCP somewhat repressed elevated glucose-mediated endothelial cell apoptosis, as confirmed by the reduced level of caspase-3, in vitro staining with caspase-8, MP, Fas, sFasL, and annexin V, which can be attributed to the hindrance of the N-terminal kinase p38/c-Jun and activation of Janus kinase 2 and its natural antioxidant properties (He et al. 2015). Zhu et al. (2017b) explained the function of MCPs in protecting the endothelial carotid artery by inhibiting biomarkers' expression, restricting endothelial dilution, and provocative exudation. They concluded that MCPs can be a possible preventive way to be safe against initial cardiovascular risks linked with T2DM.

4.4.3 Antitumor Agent and Anti-HIV Peptides

Marine fishes are a huge, multifaceted group of high protein organisms, with nearly 13,000 species, and more than 100 species are conventional tumor medicines. But just a couple of marine fish species have been artificially investigated and examined. Interestingly, most biomaterials got from marine fish have been assessed and delivered as useful functional foods, and these sorts of items have been overwhelmed by low atomic weight compounds. Some marine fish proteins have been reported recently to have a potent antitumor effect such as shark, globefishes, devil ray (*Manta birostris*), king crab, and hairy clam (*Arca subcrenata*) (Kim et al. 2001; Wang et al. 2015). Protamine and fish antibacterial peptides have also been shown to suppress tumors (Liang et al. 2010). For the study and development of new antitumor medicines, marine fish proteins are an essentially untapped option. Peptides obtained from enzymatically fabricated fish skin gelatin hydrolysate have been reported in research performed by Mendis et al. (2005) to raise levels of antioxidant enzymes in cultured human hepatoma cells with a probable function to support redox equilibrium in the cellular environment. The precise molecular mechanism that underlies antioxidant enzyme induction by MCP has not yet been explained. MCP, a combination of oligopeptides rich in proline and hydroxyproline (100–860 Da), was enzymatically hydrolyzed from the skin of chum salmon (*O. keta*), which generally causes waste and contamination in the fish preparation industry (Jang and Park 2016a; Jang and Park 2016b). The findings have shown that the long-term administration of MCPs has improved the endurance of the long-term subpopulation and delayed life expectancy. The life cycle of tumor-bearing animals in both sexes was also effectively extended by MCPs (Baum and Arpey 2005).

Marine life can become a significant source of natural anti-HIV products, due to the remarkable, entreating, and dynamic condition in which marine life forms produce different new substances. The production of marine anti-HIV agents has recently been focused on. Various studies indicated that, as a result of their therapeutic ability for treating or preventing infectious diseases, marine peptides can be used in functional foods or in pharmaceutical and nutraceutical products as anti-HIV agents (Brown and Phillips 2010). The present research reveals the prevention of HIV-1 infection of human MT-4 T cells in the marine collagen peptides, which contain APHCP (due to the Alaska pollock). APHCP inhibited the production and reverse transcriptase activity of cell lysis instigated by HIV-1IIIB and noncytotoxic performance of viral p24. However, APHCP did not suppress the HIV-2ROD infection of MT-4 cells. The following results suggested that APHC is noncytotoxic to HIV-1 and that APHCP inhibits HIV-1-pumped cell lysis, syncytious development, RT action, and viral p24 antigen formation. APHCP is a natural new peptide with a strong HIV-1 inhibitory activity and may be a therapeutic potential agent for HIV-1 therapy (Simhaee et al. 2009). These are small peptides that display anti-HIV-1 activity, rich in residues of hydroxyproline. The process of its new antiretroviral peptide was described by Jang and Park (2016b) with particular underlying mechanisms of action. The peptide is derived from the collagen of the Alaska

pollock fish. This novel peptide is highly sensitive against HIV-1 and could be a possible and encouraging therapeutic agent for HIV-1 treatment.

4.4.4 Wound Healing

The methodological technique for wound healing involves three characteristic stages: inflammation, proliferation, and maturation (Aneiros and Garateix 2004). The acceptable nutrient measure is essential to amalgamate nucleic acids, proteins, and the various components relevant to functional tissue maturation and differentiation (Liu and Sun 2014). Collagen is a characteristic cell connection, development, and differentiation substratum and covers all three stages of injury recovery. The wound healing mechanism includes many biochemical pathways, including inflammation, angiogenesis, the development of granulated tissue, reepithelialization, and wound contraction (Mehbub et al. 2014). Deposition of the collagen is an important event for the healing of wounds. In addition, the acceleration of angiogenesis is an effective method for improved recovery, particularly in incessant injuries. Angiogenesis is an integrative technique after early injury for the elimination of waste, for the delivery of necessary supplements and oxygen to the injury site, and for the advancement of granulation tissue formation. The present research investigated the injury recovery potential of MCPs, mixes of low atomic weight peptides extracted from enzymatically hydrolyzed skin of chum salmon (*Oncorhynchus keta*), and rodents using two models of injury: the excision and the incision wound type. They also intended to study the healing process and the likelihood of MCP building angiogenesis in wound tissue. Marine items would likely have many unique and potent bioactive sequences in a safe manner due to their extreme, adverse living conditions (Leal et al. 2012). Hydrolyzed collagen peptides from tilapia fish scales with a range from 0.7 to 1.3 kDa in molecular sizes will contribute to multidirectional distinguishment and osteogenic differentiation of mesenchymal stem cells from rat bone marrow, as well as to regulate the behavior of macrophages (Kreuter et al. 2003). This sponge is present in the Mediterranean Sea everywhere. They developed a wide range of secondary metabolites to defend themselves from rivals, predators, and pathogens (Nicklas et al. 2009b). Over 30 percent of the 18,000 bioactive natural marine products in pharmaceutical applications listed so far have been derived from sponges (Pozzolini et al. 2018). The biocompatibility of *Chondrosia reniformis* species with human skin has been assessed, and the use as nanoparticles for drug preparation as vehicles and coating has been described as well. Chondrosia reniformis collagen was also demonstrated to be useful as a drug preparation and nanoparticulate carrier and to assess its lack of harmfulness in human skin (Venkatesan et al. 2017; Chen et al. 2016).

Sea sponge collagen is ideal for delayed release of tablet coating and has high mechanical properties and storage stability. The prospect of using it for tissue engineering and regenerative therapeutic uses in the form of thin biocompatible membranes has also been examined (Hu et al. 2017). The recuperation, healing, and aging of the skin are another relevant area of research for which marine hydrolysates

have shown substantial results (Exposito et al. 2010). Particular interest is paid in recent years in different preclinical and clinical trials to the use of MC hydrolysates as regenerative agents for the reconstruction of the skin. Collagen hydrolysates from different sources exhibited high biocompatibility, penetration capacity, and skin-protective properties in various experimental settings. In preclinical trials, different models have shown huge defensive effects on the in vivo photoaging (Liang et al. 2010) of collagen hydrolysates of jellyfish, salmon, and Pacific cod, while Nile and chum peptides show wound healing properties (Yamada et al. 2013).

Marine fish collagen and collagen peptides have been commonly utilized as functional foods or nutritional supplements owing to their homology with human collagen structure (Girgih et al. 2013) and their safety profile (De Luca et al. 2016b). MCPs formed by the enzymatic assimilation of fish skin have been found to have various health effects primarily in two ways: metabolic issues and skin/bone repair. From the mechanistic point of view, oral administration of MCP induced, for example, endogenous collagen, to synthesize the extracellular matrix, by positively controlling the gene expression of many collagen modifying enzymes that were active in modifying and cross-linking the post-translation collagen (Zhang et al. 2011). Some in vitro studies demonstrated antioxidant properties of exceptionally low atomic weight (1–20 Da) of proline-containing MCP (Nagai et al. 2007) acting as a hydroxyl radical scavenger. Clinical examinations have demonstrated an increase in the aging parameters of the skin following oral ingestion of fish collagen peptides (Hoyer et al. 2012), which obviously demonstrated the integrity of the bioactive properties of MC hydrolysates under various pathophysiological situations of the skin. The current examination is the first to show the oral ingestion of MCP accelerates the healing of cutaneous injury using two separate rodent wound models using two injury models: the excision and the incision wound model. Tissue samples were taken on 7, 14, and 20 days after the caesarian section to determine the impacts of enzymatically hydrolyzed marine collagen peptides from the salmon skin (*Oncorhynchus keta*). At first, the fracturing power of the wounds is evidently minimal since the clotting just binds the edges together. Subsequently, the tensile strength rises rapidly with increased collagen deposition and cross-links between collagen filaments. Important improvements in the wound closure rate and increased epithelialization have been observed. The results revealed that in the 1125 g kgbw-1 MCP group, Hyp values were higher in skin wound tissues than in the vehicle group. Hyp is a derivative of proline; proline and Hyp are essential for the biosynthesis, structure, and strength of collagen (Wang et al. 2015). MCP administration by means of two different rat wound models speeds up the healing of skin injuries. Histopathological tests have established a substantial improvement ($P < 0.05$) in tensile power, hydroxyproline content, and collagen levels and an improvement in granuloma angiogenesis. It is also hypothesized that MCP can augment the initiation of antioxidants formation at an early stage of healing, which may be a significant contributor to the healing properties. Treatment of MCP rodents has led to improved wound healing, as shown by prolonged fracture closure, fibroblasts, and the production of fresh blood vessels. Angiogenesis is also an early event in the formation of granulation tissue, so the density of blood vessels was measured 3 days after injury

using a stereological procedure. Articulation of pro-angiogenic developmental factors, including VEGF and FGF-2, was tested in incisional wound tissues of different treatment groups. Both growth factors indicated the maximum improvement in MCP-treated wounds immunostained on day 3 with about 78% and 40% increase in VEGF and FGF-2, respectively, compared with vehicle-treated wounds (Bradt et al. 1999). Nagai and collaborators (Rao et al. 2011) assessed salmon collagen scaffolds for human periodontal ligament cells. Salmon (Yumuk et al. 2015) skin collagen was used for the development of porous mineralized collagen scaffolds for use in bone tissue engineering. The reassembly of collagen fibril and nanocrystalline hydroxyapatite precipitation happens concurrently in this biomimetic technique, to create a structure that is very similar to that of a natural bone (Hoyer et al. 2012). Collagen has been commonly used for the development of scaffolds due to its biocompatibility, non-toxicity, and biodegradability. Chum salmon skin was tried for its wound healing impact on a rat model isolated from MCPs. In contrast with the control group, MC was given to wound-induced rats. The findings displayed quicker wound soothing and enhanced tissue restoration at the injury site following the ingestion of MC as well as increased angiogenesis and deposition of denser and improved structured collagen fibers produced than the vehicle-treated group. In general, MC is about 60 percent purer than bovine collagen and is much safer (Bradt et al. 1999).

4.4.5 Obesity

Obesity was a major public health concern during the last century, primarily caused by unhealthy eating habits including excess calories or excess fats, poor physical action, and an inactive way of life (Rao et al. 2011). The worldwide dominance of obesity in particular almost doubled between 1980 and 2014, with about 40% of grown-ups worldwide being bulky and 13% being clinically stout. Obesity is considered to be the primary contributing element not just for nontransferable conditions, but even for different neurological and physical disorders (Yumuk et al. 2015). Type 2 diabetes mellitus (T2DM) is a particularly common type of diabetes, responsible for around 90 percent of total diabetic patients (Stumvoll et al. 2005). Patients with T2DM also face the issue of loss of insulin sensitivity. Both insulin resistance and β-cell dysfunction are key parameters for T2DM (Gloyn and McCarthy 2008). Treatment with marine salmon oligopeptides skin has shown repressed inflammation by decreasing pro-inflammatory cytokine creation in mice (Zoetendal et al. 2006). Despite several years of severe and focused study, remedies for these life-threatening disorders remain indefinable, and successful anti-obesity therapies are yet to be developed (Tschöp and DiMarchi 2017). Therefore, it is also important that experimental methods to treat and prescribe serious medical conditions associated with metabolic abnormalities be found and established. Recently, dietary obesity and the resulting complications can be avoided by natural bioactive therapy for warm-sea MCP (Astre et al. 2018). In accordance with these findings, it has been confirmed that MCPs increase glucose and insulin tolerance and

reduce the expression of T2DM rat model liver oxidative stress biomarkers and inflammatory cytokines and adipocytokines (Zhu et al. 2017a). In comparison, MCP decreases the free fatty acid levels but raises the level of adiponectin (Cui-Feng et al. 2010). The safety profile of MCP in this study seemed substantial to be greater for those who were only diabetic than for those who were diabetics with high blood pressure, suggesting that MCP would shield them from diabetes and high blood pressure by influencing different molecular levels of diabetic pathogens. A clinical trial using Nutripeptin® and Hydro MN Peptide®, two commercial MCP products, has shown that blood glucose levels are regulated and that obesity risks for patients with T2DM have been lowered to a low level. The consequences of MCP extracted from fish skin tuna on the adipogenic 3 T3-L1 breakdown and on the obese HFD-treated mouse were investigated by Lee et al. (2017). This research specifically prevented lipid aggregation during mouse differentiation preadipocyte (3 T3-L1), accompanied by a decrease in adipocyte differentiation and retention regulators expression. Furthermore, MCP blocked the lipid vacuoles aggregation in hepatocytes by palmitate; reduced the size of adipocyte; decreased overall serum cholesterol, triglyceride, and LDL; and raised serum HdL. The findings provide an insight into alternative therapeutic obesity agents.

4.4.6 Anticancer Properties

AMPs Antimicrobial peptides can be classified into two types of categories which directly operate only on bacteria and cancer cells and do not attack normal mammalian cells; and cytotoxic AMPs on bacteria, cancer cells, and normal mammalian cells (Hoskin and Ramamoorthy 2008). AMPs have the ability to be a natural source antibiotic that generally includes cationic and antitumor molecules (Rege et al. 2007). AMPs can be used in early treatment approaches and when a cream combined with CAP18 AMP is rubbed onto tumors of the exterior surface, intradermal, and epithelial skin cancer (Rodrigues et al. 2008). AMPs from the marine organisms and epithelial tumors are demonstrated to be effective. AMPs can disrupt membranes through the formation of holes, leading to cancer cell death. Epinecidin-1 cytotoxic activities have been identified with normal human cells, mouse cell lines, and multiple human carcinogenic cell lines. The findings were supported by a smooth agar experiment. Epinecidin-1 selectively destroys certain cancer cells on lower doses. Also, the results of the inhibition of necrosis and real-time PCR studies show that epinecidin-1 is not only cytolytic but also may have anti-necrosis action in cancer cells (Chen et al. 2009). The unicorn leatherjacket, belonging to the order Tetraodontiformes and family Monacanthidae, is a fish used primarily for the processing of fillets. The toughness of fish causes a lot of waste, which serves as a possible basis for the manufacture of collagen from fish. The fish peptides synthesized using Protomex and alcalase demonstrated inhibition of growth of 33.3 percent in HeLa cells but inhibition of 81.7 percent in HCT166 cells at a concentration of 1 mg/mL (Picot et al. 2006). In another analysis, bluefin and salmon skin collagen and collagen peptides displayed the highest cytotoxic activity of

65 percent and 50 percent at a concentration of 0.2 mg/mL for HepG2 and HeLa cells, respectively (Han et al. 2011). Baehaki et al. (2016) isolated collagen peptides from milkfish which demonstrated 70 percent and 18 percent inhibition in HeLa cells and HCT166 cells at a concentration of 1 mg/mL, respectively. Lower cytotoxic activity was recorded in HaCaT cells of 19.9 ± 1.9 percent at a concentration of 1 mg/ml of fish-scale collagen peptide (Subhan et al. 2017). It was inferred from the above studies that the reduction of cell growth by collagen or collagen peptides is determined by the type of cell, the type of fish, and the degree of hydrolysis (DH). In addition to these experiments, anchovy peptide 440.9 Da was observed to cause apoptosis in human U937 lymphoma cells by increasing the function of caspase-3 and caspase-8 (Lee et al. 2003). Another hepcidin TH2–3 tilapia peptide (Chen et al. 2009) and epinecidin-1 peptide grouper showed significant restraint in human fibrosarcoma cells (Lin et al. 2009). Various authors reported that the form and thickness of the collagen strands had an impact on cell growth (Subhan et al. 2017; Hassan et al. 2005). Limited reports are available on the anticancer effects of fish collagen peptides. The effect of collagen-derived abdominal skin and bluefin tuna collagen peptides on HepG2 and HeLa cells was investigated using the MTT assay (Han et al. 2011).

4.4.7 Antidiabetic and Anti-inflammatory

Diabetes is typically affected by the combination of genetic and natural causes and is characterized by the loss of insulin secretion and tolerance, which is continuously activated by the metabolic problems of weight, protein, and sugars. The in vitro antidiabetic activity of collagen peptides was tested in the α-amylase inhibition test (Kumanan et al. 2010). The collagenous gut of red porgy, common pandora, and sea bream had the most serious hindrance (Fernandez et al. 2001). Inhibitions of alpha-amylase expressed by the collagen peptides of the unicorn leatherjacket skin were about compatible with the values suggested. Hydrolyzed fish protein isolated from crucian carp (*Carassius carassius*) showed only 20.03 ± 0.89 percent inhibition of alpha-amylase at a concentration of 1.2 mg/ml, which suggested that peptides derived from fish muscle had less inhibition of alpha-amylase than collagen-derived peptides (Liu et al. 2013). As a result, the inhibition of alpha-amylase differed with the substrate, the structure of the peptide, and even the hydrolysis temperature. Zhu et al. (2010a) tested oligopeptides obtained from salmon skin for potential antidiabetic effects on rodents by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) measure. The complete procedure of collagen hydrolysates extraction is presented in Fig. 4.7 (Kumar et al. 2019).

4.4.8 Osteoarthritis

Osteoarthritis (OA), the utmost communal joint infection, is caused by ligament damage that causes joint annihilation with sternly reduced movement. Dilapidation

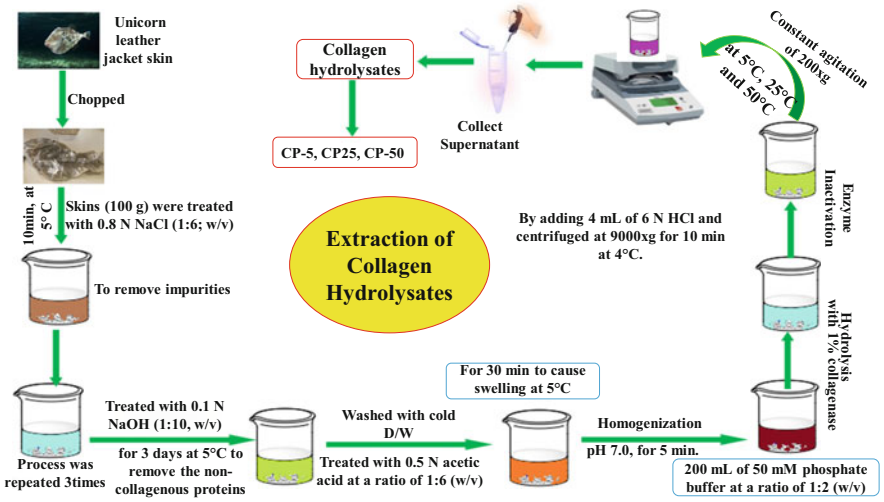

Fig. 4.7 Schematic illustration of collagen hydrolysates extraction

of the articular ligament of the affected joint is a chief component of OA. There is no compelling cure for OA as of now. The available drugs are directed at dropping manifestations such as pain and inflammation, maintaining joint mobility, and minimizing the loss of function (Henrotin et al. 2011). Glucosamine, an amino monosaccharide, is synthesized from glucose to glycosaminoglycan and helps to preserve cartilage strength, durability, and elasticity (Naito et al. 2010). Clinical examinations recommend that the use of collagen dietary enhancement can recover joint torment and mobility (Benito-Ruiz et al. 2009). Collagen was isolated from the skins of the genus Gadiformes. In addition, they looked at the effect of glucosamine and fish collagen peptide on OA. OA was triggered in 12 rabbits (12 weeks old enough) by the most cruciate tendon crosscut (ACLT). In the current study, cartilaginous tissue corruption by estimating serum levels of keratan sulfate (KS), hyaluronic acid (HA), and chondroitin sulfate (CS) 846 epitope for about one month using a reliable ACLT model and assessing the chondroprotective effect of FCP and glucosamine intake was explored. From current findings, the oral consumption of FCP or possibly glucosamine has been effectively regulated by cartilage degradation in the ACLT model. The assessment of various biomarkers for joint pain would be beneficial for the assessment of the progression of cartilaginous deterioration. In addition, the distinguished degrees of HA and CS 846 corresponded to histological observations, indicating that the HA and CS 846 figures could be worthwhile for checking the development of OA (Ohnishi et al. 2012). Thrombosis and hypertension remained the major causes of death linked with cardiovascular disease (Mackman 2008), but the search for potential therapies continues to be successful. In that context, the pulmonary collagen was utilized to make an APTA-sensor appropriate for the scientific discovery of thrombin in blood. The collagen was cross-linked to the amino thrombin aptamer developed by employing a

cross-linking agent (Derkus et al. 2016). This hybrid sensor exhibited a detection limit of 6.25 nM, well underneath the known clinical limits, indicating an intriguing potential involvement of collagen as an encouraging material for clinical thrombin testing (Derkus et al. 2016). Four novel R-purified angiotensin-converting enzyme inhibitor peptides purified from Esculentum hydrolyzed collagen were studied (Liu et al. 2016). Following oral administration of jellyfish collagen peptides angiotensin II aggregation in the kidney reduced, which resulted in a major drop in systolic and diastolic blood pressure (Zhuang et al. 2012).

4.5 Future Perspective

The current chapter should pave the way to further evaluate or re-evaluate possible marketable substitutions for collagen derived from marine sources. MCs should be a genuine substitute source of collagen. Marine species have a strong benefit in that they are believed to have a lower chance of spreading agents that induce human infection (Exposito et al. 2002) and are perceived to be much less concerned with cultural and religious issues over the human consumption of marine inferred goods. As described in the earlier parts of this study, "standard" forms of collagen, together with type I collagen commonly employed in health and foodstuffs, have been extracted and cleaned from various marine mammalian sources and non-mammals, of high purity and satisfactory yield, using traditional collagen purification techniques. However, while native, undenatured triple helix collagen is meant for use in human health goods, it can also be selected from those with maximum denaturation temperatures. It is also predicted that more structural stabilization of these marine collagens will be needed by chemical derivatives, resulting in higher temperature denaturation and greater resistance to enzymatic degradation (Zhuang and Sun 2012). A recent investigation gives a scientific logic to future uses of fish collagen as an osteogenic part in the fields of tissue engineering and stem cell treatment (Liu and Sun 2020). Marine collagen can provide real new opportunities for first-to-market opportunities, especially in the areas of health care, diversification and reduction of protection risks and cultural and religious issues, and the advancement of expertise on collagen production from different sources.

4.6 Conclusion

Marine originated proteins and peptides are turning into a significant option for medicinal applications. Numerous bioactive proteins and peptides have been developed by chemical or enzymatic hydrolysis from marine fish and have been considered as a secure alternative for the production of different items. The use of peptides and proteins extracted from marine organisms helps to reduce the environmental contamination initiated by the waste created by the fish handling industry. A great deal of consideration has been given to MC for cosmeceutical applications due to its properties for skin hydration, with low smell and better mechanical efficiency.

Furthermore, marine peptides have been thoroughly studied for medicinal applications because of their numerous organic properties, including cancer prevention, antimicrobial, photoprotective, and anti-photoactive behaviors. The poorer thermal stability of MC might be because of short residues of amino acids in comparison to mammalian collagen. The denaturation temperature of MC may be improved by the changed extraction process and the development of a composite compound for biomedical applications. Modifying the thermal stability of MC will discover a new product with the possible use of collagen in medicinal and biomedical applications and gives a premise tissue regeneration in the near future. A wide variety of collagen applications have been reported and a few more are standing to be uncovered later on. Work proceeds to find many unexplored origins of collagen and novel uses. The present state of collagen isolated from the ECM of spineless creatures and its uses in the therapeutic sector have been addressed, and some light has been shed on potential points of view of this vital marine substance. Discussion of purification methods in this analysis may be of many benefits in the production of distilled collagen from invertebrates. The various extraction techniques of marine species have been explained with descriptive imaging. The results obtained show the ability of marine invertebrates to produce new medicines, especially for bone tissue regeneration. In outline, this survey study indicates that MC can be an additional source of collagen to traditional bovine and porcine collagen. Collagen peptides extracted from MC have been appeared to display captivating biological behaviors. The prevalence of collagen in fish, starfish, wipes, jellyfish, and so on, the obstruction of certain collagen-derived peptides to gastrointestinal digestion, and their ability to enter the circulatory system indicate that MC could be a source—an intriguing bioactive peptide with exciting applications, functional food and drug distribution. A substantial amount of collagen workers have now been seen and even more are being kept up in the future. The investigation is also in transit to see the complex unexplored sources of collagen and modern applications.

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Marine Biopolymers for Gene Delivery

5

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Abstract

The marine ecosystem species biodiversity including algae, crustacean, and plant offers biopolymers such as chitin, chitosan, alginate, gelatin, laminarin, lignin, pullulan, etc. for industrial and pharmaceutical application. Marine biopolymers are an alternative source for mammalian biopolymers and play a significant role in marine waste management. In addition, plentiful properties of marine polymers, i.e., biocompatibility, lack of toxicity, biodegradability, ease of surface functionalization, antioxidant, and low cost, have steadily grown in popularity. The cationic nature of a biopolymer enables effective condensation and transfection of nucleic acid to overcome its extracellular and intracellular obstacles. Surface modification or combination of marine polymer with other polymers is a strategy to improve physicochemical properties. Furthermore processability issues, content, and physicochemical properties variation between species limit applicability of marine biopolymers, which needs to be resolved. This chapter will highlight the application of marine biopolymer for the delivery of siRNA, pDNA, and CRISPR/Cas9 for the effective treatment of various diseases.

Keywords

Marine biopolymer · Gene · Delivery · Vector · Condensation · Transfection · Chitosan

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5.1 Introduction

Gene therapy encompasses the treatment of various disease, i.e., neurological disorders, cancers, and AIDS via inactivation of disease-causing gene or introduction and replacement with healthy gene (Rai et al. 2020). Gene therapy generally involves the introduction of genetic material such as pDNA, RNA (siRNA, shRNA) and antisense oligonucleotides (AONs) (Kargaard et al. 2019), and CRISPR/Cas9 for therapeutic application (Knott and Doudna 2018). Tumor-infiltrating lymphocytes gene introduction was firstly reported in 1989 in humans (Massadeh and Al Aamery 2016). Delivery of naked nucleic acid was limited by several extracellular and intracellular hurdles which forced scientists to develop a suitable vehicle for effective delivery. Nonviral gene delivery vectors are less effective than viral vector but offer less immunogenicity, cost-effectiveness, and reproducibility, which makes them attractive vector for gene delivery (Nayerossadat et al. 2012).

Complex diversity of marine system is an indiscernible part of the ecosystem, and about 70% of the Earth is covered with ocean with 5 lakh species. Marine animals and plants are excellent sources of minerals, carotenoids, proteins, vitamins, peptides, and polysaccharides (Kadam et al. 2015). Nowadays gene delivery via nonviral polymer including marine-derived biopolymer is major exploring area. Marine biopolymers, e.g., chitosan, alginate, gelatin, lignin, pullulan, laminarin, etc., are naturally abundant in marine algae and crustaceans (e.g., crabs, lobsters, shrimps, prawns, and crayfish) (Lee et al. 2017). Marine biopolymers with unique properties, i.e., renewability, stability, being cost-effective and abundant, biocompatible, biodegradable, and nontoxicity, make them potential polymeric carriers in gene delivery (Manivasagan et al. 2017). In this chapter, we reviewed different marine polymers and their applicability in DNA, RNA, and CRISPR/Cas9 delivery to overcome the obstructions associated with nucleic acid delivery.

5.2 Challenges in Gene Delivery

Delivery of gene is not an easy job, due to various intracellular and extracellular barriers which poses strong challenges in gene delivery. The extracellular challenges involve instability in serum environment, cellular uptake, biodistribution, and rapid clearance, whereas intracellular hurdles include poor endosomal escape and off-target effects, mainly (Parlea et al. 2016). Gene delivery through intravenous or intramuscular injection experience serum environment where the presence of divalent calcium activates endonuclease enzymes which is responsible for gene degradation (Jinturkar and Misra 2011). Another complication involves the existence of tight junction between vascular endothelial cells which limits the paracellular or transcellular transport of molecules with size >5 nm diameter (Johannes and Lucchino 2018).

Cellular internalization of polyplexes is rate-limiting factor which depends on cellular interaction of the carrier and cell membrane (Lai and Wong 2018). Anionic charge on nucleic acid causes repulsive interaction with negatively charged cell

membrane which leads to the lack of spontaneous penetrability (Jinturkar and Misra 2011). Nanoparticles, carrying gene or nucleic acid as nonviral vector, having particle size approximately 1 μm, 100 nm, 50–80 nm follow phagocytosis, micropinocytosis, clathrin, and caveolae-mediated endocytosis respectively for cellular internalization (Ita 2020). Large nanoparticles >100 nm undergo RES engulfment and throw-out by RES organs (liver, spleen, lung, and bone marrow), and very small particles (8 nm or about 40 KD) are easily taken up by the kidney and suffer with short half-life and poor biodistribution due to fast renal clearance (Parlea et al. 2016; Chen et al. 2015; Jinturkar and Misra 2011).

Cationic polymeric nanoparticle (e.g., polylactic acid) showed tendency to form aggregates in the gastric environment and spleen homogenate (Lazzari et al. 2012); moreover aggregation in a circulation increases risk of embolus formation (Rai et al. 2019). After endocytic cellular internalization, another barrier involves inability to escape endosomal lysis (pH 4.5–6.2). Gene or nucleic acids taken up via endocytosis get trapped in early endosome where the existence of lysozyme degrade nucleic acids (Zhou et al. 2019). Delivery of miRNA induces Toll-like receptors (TLR) including upregulation of cytokines, IFN-α, IFN-β, IL-1B, and IL-6 via immune-toxic type 1 interferon pathway (Chen et al. 2015). Extracellular and intracellular barriers (Fig. 5.1) which limit gene delivery necessitated surge for a promising and suitable polymeric vector to overcome these obstacles, and marine biopolymers could be one possibility in this regard and may lead to successful gene delivery for effective treatment.

5.3 Marine Biopolymers

Nonviral vectors are extensively being investigated for gene delivery including polymeric nanoparticles, dendrimers, carbon nanotubes, lipid nanoparticles, etc. Polymers including marine biopolymers like chitin, chitosan, lignin, laminarin, gelatin, pullulan, and alginate have been explored by scientists for fabrication of prospective gene delivery vectors with promising results (Fig. 5.2).

5.3.1 Chitin

Chitin (cationic linear polysaccharide) is the second most abundant biopolymer comprising β-1-4 linked N-acetyl-D-glucosamine and D-glycosamine (Younes and Rinaudo 2015). The word chitin is derived from the French word *chitine* which means tunic or coverage. Chitin content has been investigated in different species like the exoskeleton of arthropods which contains 15–40% chitin whereas 20–30% chitin has been found in crustacean shells (Buschmann et al. 2013; Kurita 2006). Marine source of chitin includes mushrooms, crabs, shrimps, shells, grooved tiger prawn, Jinga shrimps, cuttlefish, scyllarid, and swimming crabs (Younes and Rinaudo 2015). Certain fish species, e.g., *Chlorurus sordidus*, *Lutjanus argentimaculatus*, and *Oreochromis niloticus*, contain low chitin concentration

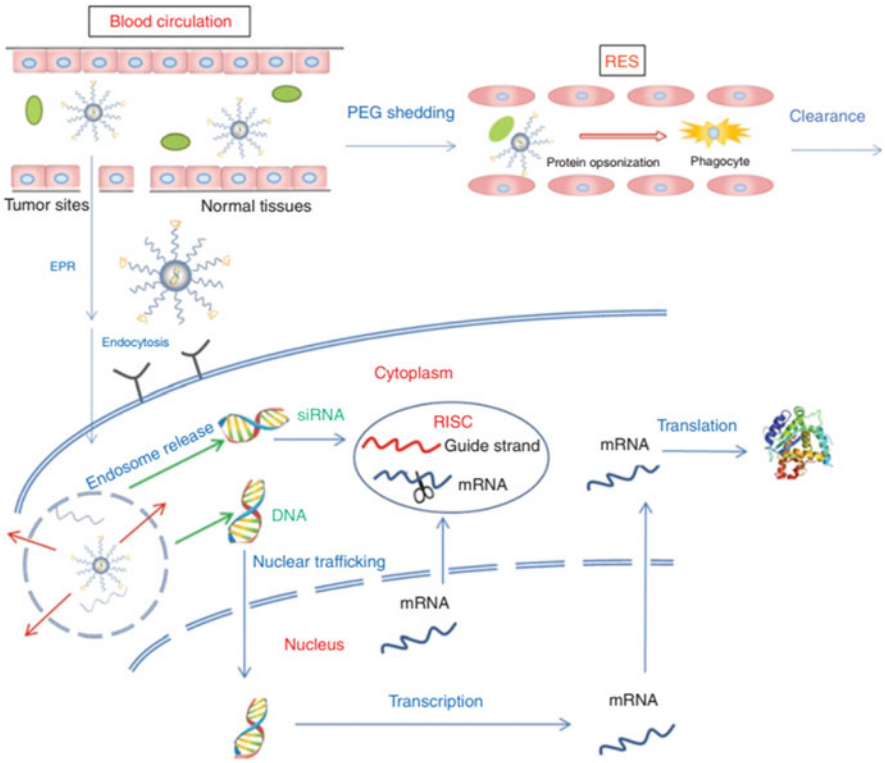

Fig. 5.1 Schematic presentation of extracellular and intracellular challenges/hurdles to be overcome for promising gene delivery (reproduced with permission from Zhang et al. 2012)

(Rumengan et al. 2017). The featured physiochemical and biological characteristics depend on molecular weight and degree of acetylation.

Chitosan (pKa close to 6.5) is deacetylated chitin prepared through the removal of R-$NHCOCO_3$ of chitin followed by alkali treatment at elevated temperature (Mohammed et al. 2017). Chitosan is having extensive relevance in the industrial field due to some important properties, such as biodegradable, antibacterial, non-toxic, nonimmunogenic,

noncarcinogenic, and biocompatibility (Manivasagan et al. 2017). Chitosan significantly dissolves in acidic solution but is insoluble in basic solution, while surface modification could improve the physicochemical property of chitosan (Negm et al. 2020). Additionally, the higher transfection efficiency could be achieved by fabricating nanoparticles using chloride, glutamate, acetate, aspartate, or lactate salts of chitosan (Weecharangsan et al. 2008). The surface functionalization of chitosan has been achieved with groups like thiol, phosphorus, glutaraldehyde, EDTA, epichlorohydrin, carboxylic acid, methacrylic acid, and benzoylated sulfate (Negm et al. 2020). Chitosan surface modification with histidine or imidazole has shown a great potential to improve proton sponge effect resulting in perfect

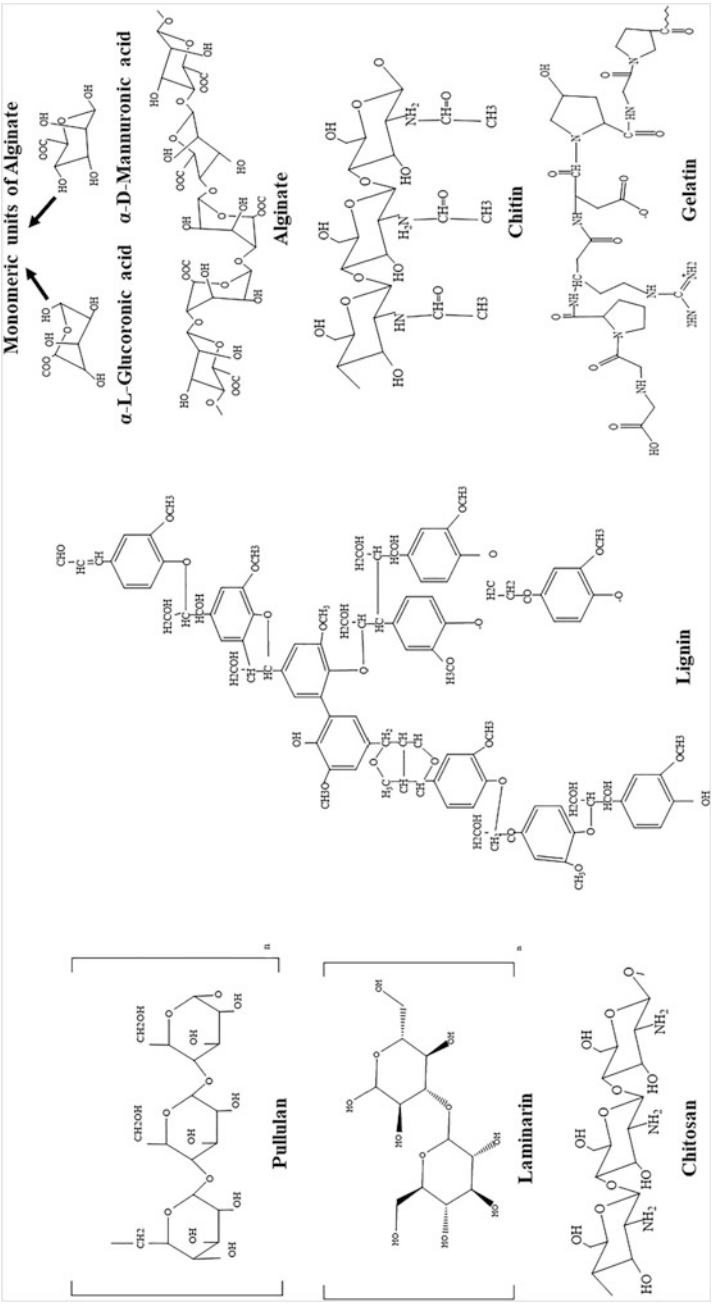

Fig. 5.2 Structure of few important marine biopolymers explored for gene delivery

endosomal escape (Chang et al. 2010). Various forms of chitosan include films, membranes, microparticles, beads, nanofibers, scaffolds, and nanoparticles (Manivasagan et al. 2017). Owing to mucoadhesive nature, chitosan interacts with mucin which reduces its clearance. Cross-linking with sodium tripolyphosphate enhances stability, shielding, and transfection efficiency of chitosan (Collado-González et al. 2019, Jonassen et al. 2012).

5.3.2 Alginate

Alginate is a linear and unbranched polysaccharide polymer (M.W. 33,000–400,000 g/mol) of 1,4-linked β-d-mannuronic acid (M) and α-l-glucuronic acid (G) residue (Szekalska et al. 2016) extracted from the extracellular matrix of brown algae (e.g., *Ascophyllum nodosum*, *Laminaria hyperborea*, *Macrocystis pyrifera*, *Laminaria digitata*, and *Laminaria japonica*) (Smidsrod and Skjakbrk 1990). Alginate has pinched much consideration due to its biodegradable, biocompatible, mucoadhesive, and hemocompatible properties (Rajaonarivony et al. 1993). Alginate marine biopolymer is naturally available in forms of alginate salt, i.e., Ca, Mg, and Na alginates (Shahabi-Ghahfarrokhi et al. 2020), and functionally characterized according to M/G ratio (Grøndahl et al. 2020). Alginates are having a block or alternating copolymer arrangement and content variation observed in different sources, such as *Pseudomonas* species lack alginate whereas *L. japonica* and *L. hyperborea* contain low and high alginate content, respectively (Rehm and Valla 1997).

The chemical structure is responsible for different characteristics of alginates, like M content stimulates cytokine production whereas cross-linking G-blocks with divalent cation Ca^{+2} is used to formulate hydrogel (Lee and Mooney 2012). Complexation of alginate with cationic polymer enhances the transfection efficiency, cytotoxicity, and shielding effect (Hu and Tsou 2014). Mixing of alginates with other materials could overcome mechanical weakness and poor cell binding problems associated with alginate. The egg box mechanism responsible for gelation includes G residue-assisted ionic bridge formation between polymer chain followed by chain-to-chain associations by calcium ion (Shahabi-Ghahfarrokhi et al. 2020). Alginate-based nanoparticle has been formulated by different methods including covalent cross-linking, self-assembly, complexation method in combination with chitosan, and emulsification method (Venkatesan et al. 2016).

5.3.3 Gelatin

Collagen frequently obtained from bovine and pork skin by acidic and alkaline hydrolysis yields denatured protein gelatin. Collagen consists of two α1 chains and one α2 chain with repeating amino acid sequence Gly-X-Y in the structure where X and Y illustrate proline and hydroxyproline units, respectively (Duconseille et al. 2015). Natural collagen exists in 29 types among them type 1 is abundant in nature (Liu et al. 2015). The major drawback associated with animal-derived collagen or gelatin except marine includes mad cow disease (bovine spongiform

encephalopathy); certain social and religious limitation of Hinduism and Islam leads to necessitate alternative source of gelatin (Coppola et al. 2020). Fishes are an important food product for the massive population and processing their waste is of major concern for the environment. The waste materials (skin, bones, and scales) are rich in collagen protein and could be plentiful alternative sources (Wasswa et al. 2007).

Worldwide production percentage of gelatin from marine source (1.5%) is quite lesser than pig (41%), bovine hide (28.5%), and bovine bones (29.5%) (Milovanovic and Hayes 2018). Kumar et al. (2017) extracted gelatin from croaker fish (*Johnius* species) skin belonging to family Sciaenidae having bloom strength of 193.4 g and pH 6.57 (1% solution). Marine gelatin obtained from collagen required shorter extraction time, but having poor textural characteristics (Kumar et al. 2017) and low imino (proline and hydroxyproline) content leads to lower melting point and gel strength (Fu et al. 2019). Generally, gelatin extracted from fish skin has low amino content than fish scales and bones (Das et al. 2017). Fish gelatin species are having variation in imino contents, functional properties, and amino acid compositions (Milovanovic and Hayes 2018). Collagen extraction from the aquatic sponge's species incorporates *Spongia graminea*, *Microciona prolifera*, *Haliclona oculata* (Gross et al. 1956), *Axinella cannabina*, *Suberites carnosus* (Tziveleka et al. 2017), *Hippospongia communis*, *Cacospongiascalaris* (Junqua et al. 1974), and *Geodia cydonium* (Diehl-Seifert et al. 1985).

Gelatin extracted from marine snail *Rapana venosa* in the family Muricidae performing acidic and enzymatic extraction using pepsin by thermal hydrolysis at temperature 60 °C shows no cytotoxicity with better cytocompatibility (Gaspar-Pintiliescu et al. 2019). Surface modification of gelatin through grafting and cross-linking with the cross-linking agents (hydroxyl, amine, and acid) can improve rheological properties. Modification of gelatin via mixing with proteins, salts, transglutaminase, glutaraldehyde, and polysaccharides is necessary for improvement of its properties so that it can compete with mammalian gelatin in terms of required properties (Lin et al. 2017).

5.3.4 Laminarin

Laminarin known as leucosin or laminaran (Kadamet al. 2014) is β-glycan food reserve material after carbon fixation in seaweed containing >30% on dry mass (Dobinson et al. 2020). Laminarin consists of 1, 3β-glucan and 1-6 β-glycosidic bond (Kadam et al. 2015). The M and G subdivision of laminarin contains mannitol and glucose terminal, respectively (Dobrincic et al. 2020). Laminarin is a plentiful biocompatible, biodegradable polymer with intestinal metabolism-modulating activity (Deville et al. 2007). Laminarin widespread applicability comprises TRAIL/DR pathway-dependent (Ji and Ji 2014) apoptosis and antiproliferation (mitochondrial pathway), allergic, anticoagulant (Kadam et al. 2014), and antioxidant activity (EC 50 value 460 μg/ml) (Choi et al. 2011) activity in the pharmaceutical field (Zargarzadeh et al. 2020). Various methods to extract laminarin are proposed, but

extraction in hot and mild acidic conditions followed by precipitation using 85% ethanol is majorly applied (Kadam et al. 2014). Laminarinase (exo- and endo-B-1,3-glucanase)-mediated hydrolysis resulted in the conversion of laminarin into glucose and oligosaccharide (Dobrincic et al. 2020).

5.3.5 Lignin

In 1813 Swiss botanist A. P. Candolle used the word "lignum" for lignin which means wood. Lignin is a natural copious biopolymer obtained from plant cell walls. Cell wall component and microfibril bind together with dense matrix which is ultimately responsible for stiffness and strength of the secondary cell wall (Martone et al. 2009). Lignin is complex, aromatic, and insoluble heteropolymer having antioxidant, biodegradability, and free radical scavenger properties (Ho et al. 2018).

Lignin contains different residues, i.e., p-hydroxyphenyl (H), guaiacyl (G), and syringyl residues derived from p-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol, respectively (Ten et al. 2014). Analysis of lignin content in marine sediment or environment is performed via chemical decomposition or thermochemoanalysis (over 600 °C). Generally, mild oxidants nitrobenzene or cupric oxide are used for chemical decomposition gives oxidants products like p-hydroxyl, vanillyl, syringyl and cinnamyl series, which are quantitatively estimated using chromatographic techniques (Li et al. 2012). Lignin extracted from marine red algae *Callarthron chellosrioides* (Martone et al. 2009), duckweed (Yadav et al. 2017), and *A. pinnata* is extensively used as an alternative in the field of biopolymer production (Setiawati et al. 2018).

5.3.6 Pullulan

Pullulan is exocarbohydrate biopolymer extracted from marine black yeast-like fungus *Aureobasidium pullulans* belonging to the family Dothideaceae having characteristic features of chlamydospores production (Bozoudi and Tsaltas 2018). Another source includes microorganisms, i.e., *Tremella*, *Cyttaria*, *Teloschistes flavicans*, *Cytariaharioti*, *Rhodotorulabacarum*, and *Cryphonectria parasitica* (Singh et al. 2017). De Bary Bernier in 1958 firstly reported pullulan as *Dematium pullulans*. Furthermore, Bender et al. in 1959 defined that pullulan structure consisted of maltotriose units (trimers of glucose connected by α-1,4 glycosidic linkage) and joint together with α-1,6 glycosidic bond (Bender et al. 1959). Pullulan generally having molecular weight range of 5000–9,000,000 g/mol is considered as exploring candidate in the field of biopolymer due to some featured properties, i.e., aqueous solubility, linear chain, flexibility, nontoxicity, and biodegradability (Kimoto et al. 1997).

Pullulan is being explored as polymeric carriers for gene delivery because of its good biocompatibility and safety profile (Prajapati et al. 2013). Biosynthesis of pullulan involves phosphoester bond formation between D-glucose and lipid

molecule mediated via UDPG. After that lipid-linked glucose structure interaction with isomaltosyl will produce isopanosyl or pyranosyl residue which further leads to polymerization of pullulan chain (Singh et al. 2017). Gibson and Coughlin (2002) performed batch fermentation process for production of pullulan from five strains of *A. pullulans* (NRRL Y-2311-1, NRRL Y-6220, NRRL Y-2567, NRRL Y-12972, and NRRL Y-12974) demonstrating extensive higher pullulan concentration in strain NRRL Y-2311-1 (Gibson and Coughlin 2002). Sulfated, phosphorylated, carboxylated, chlorinated, sulphinylethylated, and etherified pullulan are widely used for industrial application (Singh et al. 2015). Additionally conjugation or grafting of pullulan to the 1,3-dicyclohexylcarbodiimide, 4-dimethyl amino pyridine (Singh et al. 2017), diethylaminoethylamine (Sherly et al. 2020), glycidyltrimethylammonium chloride (Moraes et al. 2020), poly(L-lysine), and vinylimidazole (Caroline and Rekha 2017) provides significant improved properties and application.

5.4 Application of Marine Biopolymer in Gene Delivery

Marine biopolymers are integral part of the nonviral vector and overcome the barriers associated with naked nucleic acid delivery. Different applications of marine biopolymers in gene delivery are compiled in Table 5.1. Marine biopolymers are providing protective shielding which prevents serum degradation of naked gene. Cationic marine biopolymers like chitosan create an electrostatic connection with anionic nucleic acids providing good condensation and protection of gene (Chaun et al. 2019). Cationic biopolymer complex with nucleic acid is known as polyplex (PP), generally ionizable polyplexes having pKa value of 5–7 most preferable for DNA condensation (Prabu and Ruckmani 2017). Nucleic acid complex with polymers easily gets permeated through cell membrane due to the positive charge of biopolymer; further internalization of polyplexes generally follows endocytic pathways (Rafael et al. 2015).

Polyplex prevents endosomal degradation (pH 4.5–6.2) via promoting proton sponge effect in which cationic biopolymer prevents acidification of endosome so more proton is carried out by ATPase enzyme. Further influx of chloride ions to maintain proton concentration facilitates high ionic strength mediated osmotic swelling which is responsible for endosome rupture and escape of polyplex (Zhou et al. 2019). Additionally biocompatibility, antioxidant, and better cytotoxicity profile of marine biopolymers plays a significant role in gene therapy. The wide range applicability of different marine biopolymers for gene delivery is discussed.

5.4.1 DNA Delivery

Plasmid DNA delivery is introduced in the human body to treat inheritable disorders including severe combined immunodeficiency disease (SCID), tumor disease, cystic fibrosis, Lesch-Nyhan disease, and Gaucher disease associated with particular gene

Table 5.1 Marine biopolymer application in gene delivery

S. N.	Marine biopolymer-based nanosystem	Therapeutic DNA/RNA	Disease	Outcome/result	Reference
1.	Chitosan nanoparticle (CNP)	PKindling-Red-Mito pDNA	Cancer	CNP significantly enhances 2.8-fold of transfection efficiency than naked DNA and prevents stress	Bor et al. (2016)
2.	Lipid coated chitosan NP (lipochitoplex)	pDNA	—	Lipochitoplex (50–60 nm) having good transfection in HEK-293 cell and lipidic shield reduces the cytotoxicity and hemolysis	Baghdan et al. (2018)
3.	Chitosan-sodium deoxycholate NP	pDNA expressing Gaussia luciferase gene	Gastric carcinoma	NP exhibited good transfection in AGS, N87 cell line with >90% cell viability in N87 cell	Cadete et al. (2012)
4.	Alginate microsphere	PVAX-GFP pDNA	—	Microsphere having encapsulation efficiency 72.9–74.4% can efficiently load up to 6 μg/ml pDNA. In intestinal cells increasing the dose from 50 μg/ml to 100 μg/ml, expression of GEF was increased 13-fold	Nograles et al. (2012)
5.	Alginate nanoparticles	IL-10 pDNA	Arthritis	Reduction of IL-10 expression reduces the paw edema and also prevents other inflammatory mediators such as TNF-a, IL-1B, IL-6	Jain et al. (2015)
6.	Cholamine modified gelatin NP	AEG-1 gene target siRNA	Breast cancer	GNP with 96% loading capacity reveal the 77.25% metadherin protein inhibition after 72 h incubation	Abozeid et al. (2016)
7.	Glycidyltrimethylammonium pullulan	Calf thymus DNA	Liver disease	NP exhibited endocytotic hepatic internalization, >90% cell viability, and chain flexibility of pullulan making it hemocompatible	Rekha and Sharma (2009)
8	Pullulan hydrogel	pBUDLacZ plasmid	—	Pullulan NP having 80% and 70% cell viability on COS-7 and HEK cells, respectively, at 20 mg/ml. B-Gal high expression observed in COS-7 cells	Gupta and Gupta (2004)
9.	TMC chitosan NP	Snail specific siRNA	Prostate cancer	ChNP-CMD-SN38-siRNA NP administration exhibited sustained release up to 46 h, upregulation of E- cadherin and claudin-1 expression resulted in EMT pathway suppression, and SN38 enhances NF-KB. This nanosystem ultimately reduces the proliferation and migration of PC-3 cells	Afkham et al. (2017)

10.	Cholesterol-grafted chitosan micelles	Curcumin and siRNA	Lung cancer	Administration of micelles shows clathrin-mediated endocytosis, potential siRNA stability in serum until 30 days in lung cancer	Muddineti et al. (2018)
11.	Diethylaminoethyl chitosan ($DEAE_{15}$-CH_{230})	TNF-a siRNA delivery	Cancer	Nanoparticle effectively shows good condensation and significant 80–90% gene knockdown. MTT assay, cell viability assay performed in HELA cell reported low toxicity and 90% cell viability at concentration 1.0 μg/ml. Further PEG and folic acid conjugation completely inhibit TNF-a expression	De Souza et al. (2018)
12.	Carboxymethyl chitosan NP (CMC NP)	Anti-B-catenin siRNA	Colorectal cancer	CMC NP effectively load 70.7% siRNA and significantly 40.10% B-catenin expression reduction is observed in HT29 cells	Yan et al. (2020)
13.	Carboxymethyl dextran (CMD) chitosan NP	Doxorubicin (DOX) and IL17RB siRNA	Breast cancer	CMD-Ch-NP (particle size 114 nm) loaded with siRNA silence IL17RB gene ultimately decrease NF-KB and BCL2 expression. Synergistic effect of DOX and siRNA potentially reduces proliferation, migration, and apoptotic cell death in MDA-MB-361 cells	Alinejad et al. (2016)

deficiency (Patil et al. 2005). Chitosan is a naturally abundant biopolymer used as a nonviral vehicle for gene delivery having important properties, i.e., biocompatibility, nontoxicity, DNA condensation, and transfection efficiency. Liu et al. (2019) reported a ternary nanocomplex of folate-conjugated cis-aconitic amide-polyethylenimine with thiolated trimethyl chitosan/pDNA (187 nm) for efficient condensation of pDNA in treatment of cancer disease. In this nanocomplex quaternization, thiolation and folic acid (FA) addition improve solubility, stability, and delivery of pDNA in FA positive cells. Cis-aconitic acid (Aco) provides a protective shield to the binary complex of thiolated trimethylated chitosan (TMC-SH) conjugated with pDNA. Further charge reversible ability of FA-PEI-Aco in endosomal pH provides breakage of poor endosomal/lysosomal escape barrier due to proton sponge effect. Also, results obtained from various studies confirmed that FA-PEI-Aco/TMC-SS/pDNA exhibited low toxicity and more cellular uptake in HeLa cells. Confocal microscopy with YOYO-1 labeling demonstrated endosomal/lysosomal escape ability of chitosan NP for pDNA delivery (Liu et al. 2019).

Polyelectrolytes such as silk, hyaluronate, and dextran are used to enhance gelation properties. Chitosan nanoparticle complex with polyelectrolyte exhibited good efficacy in the delivery of human SET1 antisense (17mer DNA-based oligonucleotide) for downregulation of hSET1 gene expression which is overexpressed in malignant cells. Optimum formulation containing trimethylated chitosan (0.2–0.47 mg/ml) and hyaluronate (0.35 mg/ml), exhibited serum stability, no toxicity (up to 42 μm), and decrease in cell viability with 45% reduction in hSET1 expression in MCF-7 cell line. The scientist also reported that this nanosystem could be a good promising candidate in the colon and intestinal cancers (Baghaei et al. 2020). However delivery of pDNA is more difficult than siRNA delivery due to size and cell nucleus site of action. Co-assembled Ca^{+2} alginate sulfate nanoparticles having particle size of 270 nm and zeta potential approximately 14 mV is a suitable nanocarrier for delivery of pDNA in cancer therapy. Cytocompatibility of NP is carried out in different cells, i.e., CT26 cell, MDA-MB-231, HepG2, and neonatal rat isolated cardiac fibroblast. MDA-MB-231 cell line reveals better cytotoxicity profile, ~70% cellular uptake and ~ 30% GFP expression upon MTT assay and flow cytometer studies, respectively (Goldshtein et al. 2019). Hydrogel also has enormous potential in the delivery of pDNA which could be understood with alginate grafted PNIPAAm (Alg-g-PNIPAAm) hydrogel preparation. Hydrogel consists of 10% alginate and 90% thermoresponsive polymer PNIPAAm which provides stiffness and gelling property to the formulation, respectively. This hydrogel provides excellent pDNA release, stability, low degradation, and better toxicity profile in PC3 or MG3 cells and maintains DNA integrity. Evaluation of formulation shows the impact of molecular weight and M/G ratio of alginate on their characteristic properties. The evaluation of hydrogel demonstrates alginate 3001 grafted PNIPAAm hydrogel acts as a suitable vehicle for pDNA or RALA/pDNA complex delivery. RALA is a cell-penetrating peptide (CPP) that inhibits the direct interaction of pDNA with hydrogel. In vitro and stability studies revealed that delivery of

RLDA/pDNA is better than free pDNA due to their low burst release (~20%) and ability to maintain DNA integrity even up to 30 days (Chalanqui et al. 2019).

Oxidative stress is a major problem associated with the administration of foreign nanoparticle due to the activation of NADPH oxidase that ultimately enhances H_2O_2 level resulting in DNA degradation and cell death (Manke et al. 2013). Lignin marine biopolymer-based nanocarrier having additional antioxidant activity and their variability of functional groups (i.e., phenol, hydroxyl, carbonyl, methoxy, aliphatic hydroxyl) provides long range of new biopolymer. Lignin cored poly (glycidyl methacrylate)-co-poly(ethylene glycol) methacrylate (lignin-PGMA-PEGMA) polymer is prepared via atom transfer radical polymerization (ATRP) within the size ranging 150–250 nm at N/P ratio 10 or higher capable of efficient condensation of pDNA. ATRP enhances the lignin compatibility with other polymer, and the poly(ethylene glycol) methacrylate (PEGMA) enhances the solubility of lignin in the formulation. In vivo evaluation parameters such as relative cell viability (RCV) in H2O2 stimulated cell and the antioxidant activity in HEK293T and HepG2 cells performed, respectively. Lignin-PGMA-PEGMA graft copolymer (LG100) is most suitable due to high transfection efficiency in serum environment and antioxidant activity ($\geq$12 μg/ml), but different RCV is exhibited by nanoparticles around ~100–150% in HEK293T or 80–140% in HepG2 cells. LG100 long PGEA arm is responsible for the high binding potential of DNA and stability than LG50–30 (Jiang et al. 2015).

Polyethylenimine (PEI) polymer having excessive cationic nature is responsible for cytotoxicity, so the addition of marine biopolymer pullulan potentially reduces the cytotoxicity and modification of pullulan also enhances the transfection efficiency. Vinyl imidazole grafted pullulan PEI shows better cytocompatibility and transfection efficiency than the dextran derivative used for gene delivery. Flow cytometric analysis investigated in C6 and HeLa cells demonstrates 98.67 $\pm$ 0.32 and 94.37 $\pm$ 1.17 percentage cellular uptake and 92.6 $\pm$ 0.14 and 87.1 $\pm$ 3.11 percentage transfection efficiency, respectively. Pullulan flexibility property contributes to the efficient coating of the positive charge of PEI without affecting transfection efficiency (Caroline Diana and Rekha 2017). Furthermore, Sherly et al. (2020) grafted PEI cationized pullulan and dextran with diethyl aminoethyl methacrylate (DEAEM) ratios 10:0.01 and 10:0.005. Furthermore, studies performed demonstrate PEI cationized pullulan with DEAEM (10:0.005) exhibited efficient protection of DNA from enzymatic degradation, significant 85% cell viability, and endocytic cellular uptake in C6 and HeLa cell lines while poor internalization in L929 cells. In vivo biodistribution evaluation in BALB/c mice demonstrates no vital organs (lungs, heart, and brain) accumulation with good renal clearance within 24 h. Therefore, result expression confirmed pullulan as an efficient nonviral vector in cancer treatment (Sherly et al. 2020). So widespread applicability of DNA delivery using marine biopolymers (Table 5.1) provides effective treatment in various diseases.

5.4.2 RNA Delivery

RNA-based therapeutic agents are generally including siRNA, shRNA, microRNA (miRNA), and mRNA used for the knockdown, upregulation, or expression of a gene (Shin et al. 2018). siRNA is short double-stranded RNA ~20 bp which can silence mRNA-based expressions (Senapati et al. 2019). Sodium alginate addition in lipoplex for delivery of siRNA provides improved stability, siRNA integrity, and transfection efficiency with better cytotoxicity profile. Sodium alginate anionic part interacts with cationic lipid and the formation of auto assembly takes place. Sodium alginate containing lipoplex (Nalg-siLex) is having better particle size, zeta potential, and PDI (182 ± 9, 0.295 ± 0.049, 59 ± 1) than without alginate lipoplex (siLex) (268 ± 87, 0.747 ± 0.151, 63 ± 9) respectively. This siRNA-loaded alginate lipoplex nanocarrier administration in mice not only confirms nontoxicity but also demonstrates no significant alteration in cytokine induction and hepatotoxicity (Arruda et al. 2019).

Hypoxia-inducing factor (HIF-1α) and CD73 expression are recognized in cell proliferation and tumor vascularization, so the introduction of siRNA for the particular target could lead to efficacious treatment (Ziello et al. 2007). Hajizadeh et al. (2020) encapsulated the HIF-1α siRNA and CD73 siRNA in thiolated chitosan and trimethyl chitosan functionalized superparamagnetic iron oxide nanoparticle. Chitosan contributes in stabilization and functionalization of nanosystem; thereafter addition of HIV-1 TAT peptide (CPP) binding through S-S bond provides efficient cellular uptake of the nanoparticle. Nanoparticles are having average size of 133 nm, zeta potential +26 mV, and PDI <0.3 for effective siRNA delivery with a loading capacity of 20 μm siRNA and 50% drug release up to 36 h. The cell line used for further studies includes CT26, 4T1, B16, and F10 cells. The confocal microscopy study investigates that there is an uptake of nanoparticles by 4T1 cells and NP are able to penetrate >75% of cells. Reduction in CD73, VEGF, TGF, and FGF expression is observed after HIF-1α siRNA addition in CD73 siRNA than CD73 loaded NP alone. Angiogenesis and tumor reduction are demonstrated after chick chorioallantoic membrane assay (Hajzadeh et al. 2020).

AXL overexpression associated with non-small cell lung cancer (NSCLC) surface affects the PI3K/Akt signaling and EGFR mutation induction. Gelatin nanoparticle (quenched 208 ± 4.2 nm and non-quenched 201 ± 1.9 nm) is covalently attached with EGFR antibody, and AXL siRNA ultimately decreases the mTOR and EMT pathway with the significant enhancement in p53 expression. GAbsiAXL (antibody-functionalized gelatin nanoparticle loaded with AXL siRNA) has higher cytoplasm accumulation in HCC827 cells, serum stability up to 48 h, and ~ 70% knockdown of AXL expression with >25 nm transfected siRNA. AXL inhibition would reduce the p-AKT, p-4EBP1, and p-p7056K level and mTOR inhibition (Suresh et al. 2019).

Laminarins obtained from marine sources (algae) are natural abundant marine biopolymers. B-cell-specific Moloney leukemia (BMI) presence is observed in cellular proliferation and mammary epithelial immortality, so PEI (600 Da) functionalized laminarin nanoparticle was investigated to effectively deliver

BMI-2 siRNA. MTT assay performed confirmed nontoxicity of nanoparticle with higher cell viability (up to 96 μg/ml) and maximum transfection efficacy using NLP/siRNA mass ratio 8:1. Further investigation of the siBMI-1 to siBMI-3 revealed that siBMI-2 has a higher potential for downregulation of BMI-1 expression and apoptotic cell death than others. Remarkable loss in BMI-1 mRNA and BMI expression is observed for laminarin-based nanoparticle (Ren et al. 2015). Sadreddini et al. (2016) reported carboxymethyl dextran chitosan nanoparticle (CMD-ChNP) for codelivery of doxorubicin (DOX) and snail siRNA in HCT-116 cells for the effective treatment of colorectal cancer. Snail transcription factor (STF) is mainly responsible for the upregulation of MMP-2, MMP-9, and vimentin (EMT dependent tumor progression marker) and downregulation of E-cadherin responsible for cancerous growth inhibition. Gene silencing of STF reduces MMP-9 and vimentin, and significant cadherin enhancement ultimately leads to tumor reduction with concentration > 2.5 μg/ml of DOX-siRNA-CMD-ChNP administration within 48 h (Sadreddini et al. 2016). miRNA are smaller molecules (Collado-González et al. 2019; Coppola et al. 2020; Das et al. 2017; De Souza et al. 2018; Devillé et al. 2007; Diehl-Seifert et al. 1985) bp) regulating translation and transcription process (Ling et al. 2013).

Moraes et al. (2020) investigated modified pullulan grafted with glycidyl trimethyl ammonium chloride could be an effective vector for miRNA with significant loading efficiency (80%), cytocompatibility up to 1000 μg/mL, and good cellular uptake comparative to free miRNA or unmodified pullulan (Moraes et al. 2020). VEGF associated with age-related macular degeneration responsible for mitogenic, vascular permeability enhancement has a high binding affinity for VEGF receptor 2 (VEGFR2). VEGFR2 expression inhibitions through siRNA delivery via trimethyl chitosan and hyaluronic acid nanoparticle lead to the efficient treatment of AMD disease which is evident that marine polymer is not only limited to cancerous treatment. The addition of HA in the chitosan provides stability to the nanoplex from the anionic nature of vitreous humor. TMC-HA incorporated VEGFR2 siRNA shows no toxicity, significant cellular uptake in RPE cell (retinal tissue), 70% downregulation of VEGFR2, and retinal distribution of siRNA through intravitreal injection. CNV lesion (laser-induced) in rat reduced after administration of nanoplex. CNV lesion of control, free HA, naked VEGFR2 siRNA, and VEGFR2 siRNA loaded polyplex is estimated at 113,728.15 ± 32,289.53 μm^2, 71,346.97 ± 15.541.86 μm^2, 46,287.39 ± 19,398.42 μm^2, and 36,501.67 ± 21.739.19 μm^2, respectively (Chaharband et al. 2020).

Neurotoxicity, post middle cerebral artery occlusion, and brain injury due to iNOS-derived NO could efficiently be treated via iNOS knockdown siRNA delivery using gelatin nanoparticle (GNP). Gelatin nanoparticle having optimum particle size of 100–300 nm and cross-linked with 0.0667% glutaraldehyde follows nasal to brain delivery pathway. iNOS siRNA/GNP provides great neuroprotective potential, prolonged release up to 7 days, and cell toxicity than bare iNOS siRNA. iNOS in cortical penumbra, cortical core, and postischemic brain is responsible for the suppression of infarct volume and motor improvement (Kim et al. 2016). Therefore

studies and research work reveal that marine biopolymers have wide applicability (Table 5.1) in cancers or other diseases also.

5.4.3 CRISPR/Cas9 Delivery

CRISPR/Cas9 is pathogen-specific RNA-guided endonuclease (Lino et al. 2018). The complementary guide sequence of RNA (gRNA) binds to DNA sequence portion, and Cas9 nuclease causes cleavage of double-stranded DNA (Hussain et al. 2019). The replacement of gRNA with single guide RNA (sgRNA) also causes Cas9 directed breakage of duplex DNA strand in the presence of protospacer adjacent motif (first ~20 nucleotides of the sgRNA) where alteration and error are provoked via nonhomologous end-joining (NHEJ) or homology-directed repair (HDR) pathways mechanism (Li et al. 2018).

CRISPR/Cas9 delivery using marine polymer chitosan is a promising genome editing candidate vector for disease treatment. Surface modification of chitosan enhances the potential of CRISPR/Cas9 delivery. PEGylation of chitosan showed improved mucus penetration, no toxicity at concentration of 200–500 μg/ml, nebulization stress, and DNase protection in lung disease. PEG-C (PEGylated N-phthaloyl medium M.W. chitosan) is having a higher DNA loading capacity than PEG-OC (PEGylated N-phthaloyl oligosaccharide low M.W. chitosan) (Zhang et al. 2018). Further, Zhang et al. (2020) reported the lactobionic acid functionalized chitosan nanoparticle potentially loaded with paclitaxel and sg-VEGFR2/Cas9 plasmids represented as CLPV NP significant release at pH 7.4 and pH 5.5 sustained up to 12 h and stable until 7 days. This system relies in terms of biocompatibility and safety, no cytokine, IL-1B, and IL-6 induction reported after administration of CLPV NP. Cell lines, i.e., ASGPR positive cell and HepG2 cell, show higher cellular uptake of CLPV NP than Lipofectamine. CLPV NP exhibited >35% VEGFR2 gene silencing, alleviated side effect, reduction of neoplasm in H22-bearing mice model, and > 60% VEGFR2 protein reduction in HepG2 (Zhang et al. 2020).

Targeted delivery of the CRISPER/Cas9 has also been explored by conjugating aptamer. Liu et al. formulated biotinylated carboxymethyl chitosan NP attached with AS1411 aptamer that provides dual targeting in cancer. CRISPER/Cas9 plasmid for knockdown of CDK11 protein enhances cell apoptosis and reduction in migration or invasion. Biotinylated and aptamer functionalized CMC chitosan NP electrostatically bind to PS/CaCO3/CaP/Plasmid core and the specific binding of nanoparticle on biotin and nucleolin receptor facilitate localization of NP in biotin and nucleolin positive 492 cells without significant effect on HEK293T cells. Western blot study demonstrated inhibition of CDK11 protein expression with downregulation of VEGF, MMP-9, and survivin followed by upregulation of p53 (Liu et al. 2018).

5.5 Limitations

Naturally abundant marine biopolymer has certain limitations which became a hurdle on their applicability. The major challenge working with laminarin is that its variability in properties depends on the biological source and environmental factors. Laminaria obtained from *L. digitata* has significant water solubility, but *L. hyperborea*-derived laminarin is water insoluble in nature. Laminarin content measurement in different marine sources, e.g., *L. hyperborea*, *L. digitata*, *Saccharina latissima*, *Fucus serrate*, *Fucus vesiculosus*, *Fucus spiralis*, *Himanthalia elongata*, *Pelvetia canaliculata*, and *Dictyota dichotoma*, was found to be 0.86 ± 0.01, 0.75 ± 0.01, 0.09 ± 0.01, 0.38 ± 0.09, 0.26 ± 0.02, 0.04 ± 0.001, 0.05 ± 0.01, 0.03 ± 0.001, and 0.07 ± 0.002 percent, respectively, while lack of laminarin is detected in *C. tamariscifolia*, *A.* nodosum, and *H. siliquosa* (Graiff et al. 2016).

Alginate separation from seaweed residue is challenging because insoluble residue blocks filter and their physicochemical properties are also affected due to the use of chemicals for extraction. A large volume of water is required for alginate extraction because alginate solubility in water enhances the viscosity (Nagarajan et al. 2016). Gelatin marine biopolymer temperature and strength depend on the presence of imino acids (hydroxyproline and proline) concentration. Marine fish gelatin comprises lower imino content (22–17%) than mammalian gelatins (30%). Gelatin films of cold-water fish species have less water vapor permeability in comparison to that obtained from warm water fish and mammalian gelatins (Milovanoviand Hayes 2018). Gel strength property is investigated in marine gelatin obtained from sole, megrim, cod, hake, and squid reported to have maximum gel strength for the first two while minimum for squid (Gómez-Guillén et al. 2002). Although chitosan is most widely used marine biopolymer for gene delivery, their water insolubility limits widespread applicability and required surface modification to overcome this limitation (Zhao et al. 2018).

5.6 Conclusion

Marine crustacean, brown or red algae, fishes, and mushrooms are ubiquitous natural resources of marine biopolymers. Gene therapy including siRNA, pDNA, and CRISPER/Cas9 delivery exhibited intracellular and extracellular obstacles which limit the application and efficacious treatment of disease. Cationic biopolymer as a nonviral vector is major research exploring area having a huge potential for significant condensation and transfection of naked nucleic acid via electrostatic interaction. Proton sponge effect of cationic marine biopolymer protects from lysosomal trapped NP degradation. Marine biopolymer-based gene administration not only is limited to tumor therapy but also gives advantageous effects on neurotoxicity, age-related macular dystrophy, and lung disease. Marine source diversity is also responsible for content variation that limits its applicability in gene delivery. Moreover, specific chemical modification of marine biopolymer could be an important aspect for

properties improvement of biopolymers such as solubility and cytocompatibility and targetability upon addition of ligand or aptamer. However, the design of marine biopolymer-based nanoparticles is extensively researched by researchers with efforts to overcome limitations and improve gene delivery.

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Hydrogel Scaffolds Based on Alginate, Gelatin, and 2-Hydroxyethyl Methacrylate for Tissue Regeneration

6

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Abstract

The design of bioactive scaffolding materials with favorable properties is paramount for successful application in biomedical engineering. Polymeric hydrogels attract significant attention as leading candidates for scaffold engineering due to their specific compositional and structural similarities to the natural extracellular matrix. The ability to control porosity, surface morphology, and size of hydrogel scaffolds has created new approaches to overcome various issues in tissue engineering such as vascularization, tissue architecture, and simultaneous multiple cells seeding. This review imparts an overview of hydrogel scaffolds based on synthetic and natural polymeric components (alginate, gelatin, and 2-hydroxyethyl methacrylate). We made hydrogel scaffolds with unique properties. Their in vitro and in vivo biological response, morphology, mechanical properties, porosity, hydrophilicity, and degradability were tested to find optimal patterns of tissue regeneration.

Keywords

Hydrogel scaffolds · Alginate · Gelatin · 2-Hydroxyethyl methacrylate · Tissue regeneration

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6.1 Tissue Regeneration Engineering and Polymeric Scaffolds

Tissue regeneration and engineering received significant scientific interest (curiosity) in the last decades and they can improve the levels of classical treatments. A biomaterial as a "temporary matrix that promotes a specific environment and architecture for tissue growth and development" is defined in the field of tissue engineering (Roseti et al. 2017).

The principal therapeutic platform for regenerative medicine is tissue engineering. The expansion of substitute products using biomaterials that can regenerate and repair diseased tissues and organs is a significant issue of new technological approaches. Therefore, these medical devices are fabricated to overcome the limited regeneration capacities of human tissues and organs.

The regenerative medicine field is dedicated to the general goal of damaged organs and tissues repairing and regenerating. Patient living cells isolation, their in vitro spreading inside polymeric scaffold, and reimplantation of tissue-mimick structures in the patient are the courses of the regenerative process (Langer and Vacanti 1993). The versatile properties of polymeric biomaterials, especially hydrogels, have found significant application in regenerative medicine as scaffolding materials into which cell cultures are introduced and serve as devices for the delivery of cells and genes (Slaughter et al. 2009). These biomaterials may be made biocompatible and exhibit adjustable mechanical properties and degradability. They are incorporated with biological signs to be able to carry out the processes of adhesion, migration, and proliferation of cells and growth factors, to enable the binding sites of peptides or cytokines. The biomimetic polymeric biomaterial thus created can mimic an extracellular matrix (ECM) environment.

Regenerative engineering aims to advance complex tissue revitalization processes and biological systems and encompasses the fields of stem cell science, advanced biomaterials, and developmental biology (Laurencin and Khan 2012). Polymeric hydrogels can be fabricated into 3D mimetic scaffolding for cell growth support thanks to their resemblance to a native extracellular matrix making them attractive biomaterials for regenerative engineering. The application of nano- and microtechnologies leads to the possibility of managing the properties and functionalities of hydrogel materials. This contributes to the biomimetic production of more refined patterns and architectures. Thus, our scientific perspectives expand to the level of nanoscale cellular interactions.

Most human tissues have a limited capacity for regeneration. Severe tissue damage due to trauma, degenerative diseases, or congenital abnormalities can lead to irreversible disability or death. Therefore, patients with serious tissue damage, in that case, depend on organ transplantation. Despite the increased volume of organ transplants, there is a limitation in the possibilities of recipients and donors of organs. Then tissue engineering and regenerative medicine take over the ″role″ (Amado et al. 2017; Vacanti and Langer 1999; Mason and Dunnill 2008).

The ideal scaffold should mimic a native extracellular matrix (ECM) and direct stem cells to regenerate functional tissues. In that sense, hydrogel materials are one of the most beneficial biomaterials with tunable water/fluid level, a high degree of

biocompatibility, and simple adjustability to restore ECM (Slaughter et al. 2009). Various kinds of hydrogels with favorable properties are created to fix different type of tissues (Huebsch et al. 2010; Engler et al. 2006). Biophysical cues, like porosity, elasticity, and degradability, can be introduced into hydrogel scaffolds in a specific spatiotemporally manner to systematically regulate the cell's behavior (Guvendiren and Burdick 2012; Yang et al. 2016). Advanced chemical strategies are used to improve biocompatibility and achieve hydrogel functionality (Amado et al. 2017; Phelps et al. 2012).

Spatially patterned hydrogel scaffolds with nanoscale biophysical and biochemical cues are used to evaluate cell behavior (Yang et al. 2016; Wade et al. 2015). Nanofiber-based hydrogel that mimics ECM with adaptable architecture and mechanics is synthesized in the interaction of advanced polymer processing technologies. In this way, the sensitivity of cells to mechanical stimuli as well as subsequent responses can be tested (Baker et al. 2015).

The potential of regenerative medicine is based on the regeneration and replacement ability of diseased tissues and organs (Atala 2008; Mendelson and Frenette 2014). Researches in the field of regenerative medicine have results that indicate that can be reported successful regeneration and replacement of many tissues and organs, including skin, heart, kidneys, and liver, as well as the potential to even improve some birth defects (Amado et al. 2017; Bailey et al. 2014; Knoepfler 2015; Tang et al. 2016).

Powerful biomaterials, mainly combinations of scaffolding, growth factors, and stem cells, have the function of replacing damaged tissue and showing that they function as original tissue or have the ability to stimulate native tissue regeneration to establish successful regenerative medicine platforms (Amado et al. 2017; Gao and Cui 2016; Guan et al. 2017). These platforms focus on tissue environmental changes due to the incorporation of exogenous materials and biological factors with the sole purpose of accelerating and promoting the body's healing process. Extracellular matrix materials and biomimetics have been used for more than a decade, more than just providing physical support (Amado et al. 2017; Alves da Silva et al. 2017; Goncalves et al. 2017; Pina et al. 2017). Materials and biomimetics can in their own way stimulate the regeneration process, but they can also be biomolecules on their own that affect cell growth (Guan et al. 2017). In addition to the necessary physical support to the cells, it is advantageous for the scaffolding biomaterial to include biological signs or signals that enhance or improve tissue regeneration and performance (Dzobo et al. 2016; Evans et al. 2010; Sadtler et al. 2016). Different tissues possess the different regenerative capacities. So, some do not need the addition of cells, but only biomaterial and biological agents. Other tissues have limited regeneration capacities and need biomaterial, biomolecules, and cells for regeneration. Cartilage and cornea are tissues and organs with limited or no regenerative capacity, while the liver and lungs have a high regenerative capacity (Amado et al. 2017; Atala 2008; Atala 2012; Kotton and Morrisey 2014).

Native tissues have a wide range of physical characteristics, of which elasticity and permeability stand out. For example, human tissue shows tissue-specific elasticity in the range of about seven orders of magnitude, starting at only 167 Pa for breast

tissue (Guan et al. 2017; Paszek et al. 2005), to as high as 54 GPa for hard tissue (Choi et al. 1990). There are also various optimum pore sizes of implants for inducing regeneration of different types of tissues: 5 μm in pore diameter for vascularization (Brauker et al. 1995), 5–15 μm for fibroblast ingrowth (Klawitter and Hulbert 1971), 20–125 μm for adult skin regeneration (Yannas et al. 1989), and 100–350 μm for bone regeneration (Annabi et al. 2010). Cells can recognize mechanical signals and through mechanical transduction reach further processes differentiated into different families (Guan et al. 2017; Huebsch et al. 2010; Engler et al. 2006). Hydrogel materials can contain biophysical responsiveness in a controlled way to control cellular manner, mimicking these physical characteristics on a micro and nano scale. This approach gives an obvious understanding of the mechanism of cell development and their reactions in various diseases (Guan et al. 2017).

One of the three most important elements in the basic concept of regenerative medicine is scaffolding materials. They form the basis of regenerative medicine engineering. Surgical procedures involving the use of scaffolds as medical devices are performed daily to replace or repair tissue damaged by disease or trauma. Highly porous scaffolding biomaterials combined with the body cells can form templates for tissue regeneration, which lead to the growth of new tissue. The scaffold has a prominent role in the design and fabrication of 2D and 3D models, including surface ligands and molecular architecture, nanoparticle-cell interactions (Amado et al. 2017).

Scaffold modeling is a crucial part of tissue engineering and regenerative medicine. A fundamental tool to guide tissue formation both in vitro and in vivo should be an optimal designed 3D scaffold. Properties like high surface-area-to-volume ratio, porosity, pore size, pore design, pore interconnectivity, permeability, and degradation should be taken under consideration when designing scaffold material for various and tailored applications. These permit a desirable biological network for cell migration, nutrient transportation, and therefore the mechanical stiffness, and strength are therefore obtained (Budama-Kilinc et al. 2017; Parker et al. 2002; Fang et al. 2005). Growth factors and drug release process should even be considered to attain optimized tissue growth as scaffold degraded. Moreover, some researches have shown the advantages for tissue regeneration of using curvature and concave surfaces compared to convex and planar ones (Zadpoor 2015). Two scaffolding design approaches may be used to fulfill the aforementioned demands. The first one is predicated on the native tissue. The second relies on the unit's digital cell model, both addressing tailored scaffolding geometry. The geometry obtained can then be applied to computer-aided engineering studies to optimize the performances of the tailored bioengineering scaffold (Amado et al. 2017).

The artificial scaffold is made and its properties are optimized according to the complex structure of the tissue. The scaffolding designed in this way should be suitable for cell adhesion, proliferation, and ECM formation. This is how the ECM recovers. Medical devices should be designed to create, induce or congest tissue reactions (Amado et al. 2017; Haycock 2011). The perfect scaffolding should have optimal and favorable biocompatibility, biodegradability, mechanics, interconnection of pores of appropriate sizes that will accommodate cells, as well as an optimal

exchange of nutrients and waste products with its three-dimensional space for cell growth in an organized manner (Amado et al. 2017; Seol et al. 2013). Tailoring biomaterials for enhanced biofunctionality can be accomplished by employing a kind of approaches that involve the introduction of chemical, topographical, or mechanical cues via top-down or bottom-up approaches (Camarero-Espinosa and Cooper-White 2016). It is very important to choose favorable biomaterials, as well as techniques and methods of obtaining them. There is a whole range of natural and synthetic components used for scaffold syntheses such as biometals, ceramics, bioactive glass, phosphates, and polymers. Bioactive ceramic scaffolds are favorable for bone tissue regeneration due to their biological functionality. However, ceramics as scaffolding materials is restricted owing to difficult processability and inherent brittleness (Seol et al. 2013). Rezwan's group (Rezwan et al. 2006) showed that conventional material processing methods are adapted and extended for the incorporation of inorganic bioactive part into porous and interconnected 3D polymeric networks (Amado et al. 2017). Advanced biomaterials are hybrids with adjustable bioactivity and biodegradability (Rezwan et al. 2006). Addressing these issues, research has focused on the mechanical testing of polymer-bioglass composites. Consequently, scaffolds of identical density are compared and it has been confirmed that the addition of bioglass reduces the elastic gradient and relieves tension. Therefore, the highest content of bioglass seems to have a beneficial effect because it does not significantly impair the mechanics of the scaffold and improves bioactivity (Fiedler et al. 2015). The creation of biodegradable, bioactive, "living" composite biomaterials loaded with biomolecules, which is characterized by great adaptability to the biological environment (Rezwan et al. 2006), is a promising approach. Biomolecules are added to speed up the healing process, and they are sensitive to elevated temperatures and extreme chemical conditions. Therefore, their loading during scaffolding production is not easy. Another interesting approach is the immobilization of proteins and growth factors by functionalizing the scaffold surface in the postprocessing phase (Amado et al. 2017; Cosson et al. 2015).

"Soft" material routes like sol-gel processing may be a platform to include biomolecules during scaffold fabrication. But, highly porous, interconnected pore structures for bioactive organic/inorganic hybrids have not yet been achieved, which is important for scaffolding application. Further, an important issue is a reason for the local impact of growth factors on the cell and tissue systems, especially long-term effects (Amado et al. 2017; Rezwan et al. 2006). The foremost, crucial parameters that determine the performance of a designed implant are mechanical characteristics which depend on the process and structural characteristics of the biomaterials. In such a way, desired mechanical properties can accomplish by modifying the structural characteristics of a biomaterial. Cell biological behavior testing is required to check biocompatibility and bioactivity after surface modifications (Amado et al. 2017; Haycock 2011). The study of the interactions of biochemical and geometrical cues on stem cell differentiation and alignment should be also considered. The potential to spatially control stem cell orientation and differentiation toward multiple phenotypes simultaneously, i.e., myocyte, tenocyte, and osteoblast allow in vitro cells grown to more closely mimic aspects of native

structure organization (Amado et al. 2017;Ker et al. 2011). Although the precise mechanism behind geometry-induced cell alignment is presently unknown, cells alignment observed on fibers may likely be attributed to a mix of factors including physical space constraint and relative stiffness of the underlying substrate; ultimately effecting changes in both cell spreading and cell stiffness, cells could also be predisposed toward a selected orientation through the modulation of mechanotransduction pathways via cytoskeletal rearrangements (Amado et al. 2017; Sun et al. 2004).

A more robust choice to create graded structures that resemble the biological interface is to form multilayer scaffolds as combinations of several biomaterials. The newest technologies deal with the development of multilayer scaffolds and the controlled release of bioactive agents to promote in situ regeneration of biological tissues and aim to perfect the available traditional treatments. Long-term evaluation is indispensable with a special focus on studying the biological and mechanical properties of the designed tissues to verify the potential of those novel approaches (Amado et al. 2017; López-Ruiz et al. 2016).

The scaffold design should enable the creation of a bioactive dynamic system, which will be a favorable environment for cells. One of the types of scaffolds is nanofibers that are obtained by the technique of surface modification, which contribute to an improved understanding of in vitro and in vivo cellular behavior. This approach implies the interconnection of knowledge of science and technology (Amado et al. 2017; Cheung et al. 2015; Kariduraganavar 2016).

In organ engineering, it is important to take care that the regeneration of functional tissue takes place, whereby it is necessary to achieve cell viability and differentiation, stimulate proliferation, the direction of modulation, and the rate of cell migration and cell adhesion regulation (Amado et al. 2017). Cell viability is determined through morphological changes or membrane permeability, as a physiological condition based on cell culture staining. Cells are cultured on a three-dimensional biocompatible scaffold. This is followed by a process of degradation and resorption at a projected rate whereby soft and hard tissues are regenerated in vitro or in vivo conditions (Amado et al. 2017; Hutmacher 2001).

The bioengineering approach in scaffolding design in vivo is guided by the conditions of oxygen diffusion and the flow of nutrient components so that the thickness of the scaffold is adjusted to several hundred microns (Amado et al. 2017; Cosson et al. 2015). Great care must be taken with scaling in the transition to clinical applications from in vivo testing. An important topic is the process of tissue vascularization. Good blood circulation to the tissues leads to a favorable flow of nutrients and other components and leads to the proper integration of cells and the healing process. The latest biocreation techniques, especially when it comes to bulkier medical devices scaffolds, lead to more advanced solutions to vascularization issues (Amado et al. 2017;Lim et al. 2017).

The most widespread biomaterials used for the design of scaffolding and medical devices in regenerative engineering are polymers (Budama-Kilinc et al. 2017; Piskin 1994; Ji et al. 2006). The properties of materials that recommend them to be used toward biomaterials are composition, hydrophilicity, swelling, surface energy,

adhesiveness, the possibility of degradation, and erosion mechanism. Polymeric scaffolds have emerged as the biomaterials of choice due to their specific properties that can be adjusted to different synthesis processes and techniques (the length of polymeric chains is a key factor that further determines all properties relevant to biomedical applications). Due to all the above, it is possible to very finely achieve the most favorable bioactivity and biocompatibility of scaffolds. Research on the development of artificial skin and cartilage has been conducted in that direction (Eaglstein and Falanga 1998), bone and cartilage (Boyan et al. 1999), liver (Mayer et al. 2000), heart valves and arteries (Mayer et al. 1997), bladder (Oberpenning et al. 1999), pancreas (Tziampazis and Sambanis 1995), nerves (Mohammad et al. 2000), corneas (Germain et al. 1999), and various other soft tissues (Diedwardo et al. 1999).

One of the scaffolding polymeric materials classifications is synthetic, biologic, degradable, or nondegradable concerning their application (Budama-Kilinc et al. 2017; Ramakrishna et al. 2001). The behavior of polymers results from their properties-composition, structure, conformation, and arrangement of basic repeating units.

Natural polymers are often reviewed as the first biodegradable biomaterials used clinically (Nair and Laurencin 2007). Natural materials are considered to be bioactive properties and thus lead to better interaction with cells. This leads to improved cell performance in the biological system. They are included in natural polymers proteins (silk, collagen, gelatin, fibrinogen, elastin, keratin, actin, and myosin), polysaccharides (cellulose and derivatives, amylose, dextran, chitin, and glycosaminoglycans), or polynucleotides (DNA, RNA) (Yannas 2004).

Synthetic polymeric materials have proven to be very favorable in the function of restoring the structure and performance of damaged tissues. Their properties essential for this purpose such as porosity, degradation time, and mechanical properties can be adjusted in various ways and adapted to specific applications. One of the advantages of synthetic polymers is that they are often cheaper than biological. Their production takes place in large uniform batches, and the shelf life is longer. Also, a wide range of commercially available synthetic polymers possesses physicochemical and mechanical properties that mimic the properties of biological tissues. Synthetic polymers represent the largest group of biodegradable polymers, and that they can be produced under controlled conditions. Their properties are predictable and repeatable, and they include strength to different types of loads, multiple module types, and degree of degradation (Gunatillake et al. 2006). Polylactides and polyglycolides (homo- and copolymers) are among the foremost commonly used synthetic polymers in tissue engineering (Ma 2004). Polyhydroxyalkanoates belong to a category of microbial polyesters and are widely investigated for tissue and regenerative engineering applications (Chen and Wang 2002).

Bioactive ceramics and specified silicate and phosphate glasses (bioactive glasses) and glass-ceramics (apatite-wollastonite) can react with physiological fluids and through cellular activity form firm interconnections to hard and in some cases soft tissue (Hench 1998). However, the biocompatibility and biodegradability of these compositions are often insufficient, diminishing their potential use within the clinical part. By combining synthetic and natural polymeric components in different

ways or by using composite materials that improve the properties of scaffolds, such phenomena are promoted and thus enable controlled degradation (Cascone et al. 2001) and improve biocompatibility in tissue engineering (Ciardelli et al. 2005). Especially, degradable polymers and bioactive inorganics in synergy represent the platform to achieve mechanical and biological performance in hard tissue (Roether et al. 2002).

In the last 10 years, hydrogels as three-dimensional polymer networks that have taken precedence as leading biomaterials in the field of tissue engineering, where they are used as scaffolding to guide the growth of new tissues. The potential impact of hydrogel materials in the biomedical field has been significantly increased based on the possibilities of tailored design, especially those of biodegradability. This is followed by a very important area of the system for the controlled release of active agents (Budama-Kilinc et al. 2017; Cabodi et al. 2005). When considering properties essential for tissue regeneration-biocompatibility, cell-controlled degradability, and internal cell interactions, naturally occurring polymer-based hydrogels have an advantage. As for obtaining this type of polymer, there is variation in batches, but their structure and functional properties are precisely controlled. Hydrogels possess a structural identity similar to the macromolecular based constituents within the body and highly biocompatible behavior (Jhon and Andrade 1973). Hydrogels are made of synthetic or natural polymers or combinations thereof, where covalent or noncovalent bonds are established by the cross-linking agent(s) (Hoffman 2001). For hydrogels to be used in tissue engineering, they must meet a whole range of standards to perform their role and contribute to the formation of new tissues. These criteria refer to the classical physical parameters (degradation and mechanics) similarly to biological parameters (cell adhesion). It is commonly presumed that the degradation rates of tissue scaffolds must be matched to the speed of varied cellular processes to optimize tissue regeneration (Hubbell 1999; Lee and Mooney 2001). Therefore, the degradation behavior of biodegradable hydrogels should be defined, reproducible, and tunable via hydrogel chemistry and structure. Biocompatible hydrogels are currently employed in cartilage healing, bone regeneration, wound dressings, and as drug delivery systems (Peppas and Khare 1993). Hydrogel loaded with growth factors can act to support the development and differentiation of cells within the newly formed tissues (Tabata 2003). Hydrogels are often favorable for promoting cell migration, angiogenesis, optimal water/fluid content, and rapid nutrient flow (Bryant and Anseth 2001). Hydrogel scaffolds are the subject of intensive research in the engineering of replacement connective tissues, primarily because of their biochemical similarity with the highly hydrophilic glycosaminoglycan components of connective tissues. Hydrogel-forming polymers of the natural source are collagen (Wallace and Rosenblatt 2003), gelatin (Kim et al. 2004), fibrin (Eyrich et al. 2007), hyaluronic acid (Solchaga et al. 2002), alginate (Kong et al. 2003), and chitosan (Francis Suh and Matthew 2000). Synthetic polymers are polylactides (Schmedlen et al. 2002), polypropylene fumarate-derived copolymers (Behravesh and Mikos 2003), polyethylene glycol derivatives, and polyvinyl alcohol (Bryant et al. 2004).

Alginate is a naturally derived macromolecule; polysaccharide extracted from brown algae, such as *Macrocystis pyrifera*, *Laminaria hyperborea*, *Ascophyllum nodosum*. Naturally, it has an anionic character and consists of β-D-mannuronic acid and α-L-guluronic acid repeating units (Das and Subuddhi 2020). It manifests vital properties like biocompatibility, biodegradability, nontoxicity, nonimmunogenicity, and transparency. Gelation is accomplished easily, which enables alginate to be widely used in biomedical applications such as drug delivery, cell encapsulation, wound healing, and tissue engineering (Das and Subuddhi 2020; Lee and Mooney 2012). A modified version of alginate is sodium alginate which is a salt of alginic acid. Alginate is polymeric material with good scaffold-forming properties that can be useful to treat the loss or failure of organs.

Alginate hydrogels are also being actively researched for their beneficial properties to mediate the regeneration and engineering of various tissues and organs, including skeletal muscle, nerve, pancreas, and liver (Lee and Mooney 2012). Current strategies for skeletal muscle regeneration apply to cell transplantation, growth factor delivery, or a fusion of both approaches (Saxena et al. 1999; Levenberg et al. 2005), and alginate hydrogels have found potential in these platforms. The combined delivery and release of vascular endothelial growth factor and insulin-like growth factor-1 from alginate hydrogels were used to regulate both angiogenesis and myogenesis (Lee and Mooney 2012). The localized and sustained delivery of both growth factors led to significant muscle regeneration and functional muscle formation, because of cell activation and proliferation, and cellular protection from apoptosis by the released factors (Borselli et al. 2010). Long-term survival and outward migration of primary myoblasts into damaged muscle tissue in vivo from RGD-alginate hydrogels were significantly improved by the sustained delivery of hepatocyte growth factor and fibroblast growth factor2 from the hydrogels (Hill et al. 2006a, b). This led to extensive repopulation of host muscle tissues and enhancement of the regeneration of muscle fibers at the wound site (Hill et al. 2006a, b).

Alginate hydrogels have also been tested as biomaterials that can act to repair the central and peripheral nervous system. Alginate-based highly anisotropic capillary gels, introduced into acute cervical spinal cord lesions in adult rats, were integrated into the spinal cord parenchyma without significant inflammatory responses and directed axonal regrowth (Prang et al. 2006). Alginate gels, covalently cross-linked with ethylenediamine, can be useful to restore a 50 mm gap in cat sciatic nerves (Lee and Mooney 2012; Hashimoto et al. 2005) and promote the outgrowth of regenerating axons and astrocyte reactions at the stump of transected spinal cords in young rats (Kataoka et al. 2004). Alginate gels are also used as glue for the repair of peripheral nerve gaps that could not be sutured (Suzuki et al. 2000).

Alginate has shown great use and potential as a biomaterial for several biomedical applications, for wound healing, drug delivery systems, in vitro cell culture, and tissue engineering (Lee and Mooney 2012). The foremost attractive features of alginate for these applications include biocompatibility, mild gelation conditions, and easy modifications to make alginate derivatives with new properties. There are documents on the safe clinical use of alginate as a wound healing and dressing, as well as pharmaceutical compositions. It can be safely implanted in a variety of

applications, including islet transplantation for type 1 diabetes (Soon-Shiong and Heintz 1994) and chondrocyte transplantation for urinary incontinence and vesicoureteral (Diamond and Caldamone 1999; Kershen et al. 2000). A chemically modified alginate can be also widely used as a carrier to advance periodontal regeneration (Sculean et al. 2001). However, alginate gels have limited mechanical stiffness and more general physical properties. An ongoing challenge is matching the physical properties of alginate gels to the necessity in an exceedingly particular application. Consideration of the range of various available cross-linking strategies, using molecules with various chemical structures, molecular weights, and cross-linking functionality will often yield gels suitable for every application. Determined research concepts evaluate cell encapsulation strategies (Lee and Mooney 2012). But, covalent cross-linking reactions can cause toxicity to the cells to be encapsulated, and an appropriate choice of cell-compatible chemical reagents (initiator, activator), and thorough removal of unreacted components and by-products will likely be needed in those applications (Lee and Mooney 2012; Smeds and Grinstaff 2001).

Dynamical control over delivery can potentially improve the drug's safety and effectiveness and assure new therapeutic platforms. On-demand drug release from alginate gels in response to external cues like mechanical signals (Lee et al. 2000) and magnetic fields (Lee and Mooney 2012; Zhao et al. 2011) can be exploited to design active depots of the many drugs, including therapeutic cells. The introduction of appropriate cell-interactive features to alginate is additionally crucial in many tissue engineering applications. The kind of adhesion ligands and their spatial organization in gels are key variables, as they can adjust cell phenotype and also the resultant function of regenerated tissues. While RGD peptides are extensively exploited thus far as a cell adhesion ligand, multiple ligands and/or a mix of ligands and soluble factors could also be required to properly produce replacement tissues and organs.

Gelatin is a natural polymer obtained by partial hydrolysis of collagen, which is considered to be nonimmunogenic, biodegradable, easy to process, and biocompatible for clinic use (Mohammadzadehmoghadam and Dong 2019; Lien et al. 2009; Aldana and Abraham 2017; Babitha et al. 2017). Most ECM is made of gelatin and carbohydrates. However, gelatin is rarely used as the sole constituent owing to its high brittleness, and thus needs to be functionalized using cross-linking, grafting, and blending methods (Mohammadzadehmoghadam and Dong 2019; Hersel et al. 2003; Zhang et al. 2006; Wongputtaraksa et al. 2012; Taddei et al. 2013; Poursamar et al. 2016).

Gelatin is often made through thermal and chemical denaturation of collagen with pronounced properties of nontoxic, nonirritant, and biodegradability, likewise nearly as good living body compatibility (Bigi et al. 1998; Bajpai and Kankane 2007). Gelatin contains an Arg-Gly-Asp (RGD) sequence which improved cell adhesion and migration (Huang et al. 2005). It possesses a high degree of biological functional groups and potential for tissue scaffolding applications. Gelatin can be used in pharmaceuticals, wound dressings, and adhesives in clinics because of its good

cell viability and lack of antigenicity. Improved formability and low costs allow selection and mass production (Kim et al. 2005).

Gelatin can be also used as a coating agent to enhance cell attachment as a technique of vascular tissue regeneration (Nair and Thottappillil 2015). Gelatin shows gel-forming, thickening, emulsifying, and foaming proprieties. The mechanical ones depend on the supramolecular structure (Kozlov and Burdygina 1983). Syntheses of anionic or cationic gelatin are based on extraction conditions and fundamentally for filling molecules through electrostatic interaction. Since gelatin stability at high temperatures and a wide range of pH allow synthetic polymers to be grafted on the gelatin backbone. The methods are known as "grafting from", polymerization on the functional surface of the substrate, "grafting to," grafting of a fully functionalized polymer to the substrate, or "grafting through" (Sadeghi and Heidari 2011).

Gelatin-based formulations are employed in biomedicine and therapeutic agent controlled delivery (Sajkiewicz and Kołbuk 2014). Polyethyleneimine-functionalized gelatin can increase transfection efficiency thanks to positive nanoparticles that facilitate DNA loading (Kuo et al. 2011). It indicates the possibility to use hydrogel as vectors for gene controlled delivery.

Methacryloyl group-grafted gelatin, also known as gelatin methacrylate (GelMA) had been used for the microfabricated blood vessel. Gelatin electrospun fibers loaded with antibiotic drugs show a strong antibacterial activity but this gelatin has to be reinforced with materials such as graphene oxide and boron nitride, to improve poor mechanical proprieties (Bakhsheshi-Rad et al. 2017; Nagarajan et al. 2017; Nagarajan et al. 2016a, b).

Gelatin copolymer acts as a natural cell surface that reacts under mechanical stimulation when the electrospinning process is used to create tubular scaffolding and allows obtaining a similar morphology as the native ECM structure (Thomas and Nair 2012). Panzavolta's group obtained electrospun gelatin nanofibers cross-linked with genipin (low-toxicity agent) which reduces the extensibility of the electrospun mats, producing appreciable improvements in elastic modulus and breaking stress (Panzavolta et al. 2011). Long-term studies on cross-linked electrospun gelatin are still in progress to confirm its efficacy as a biomaterial scaffold for blood vessel tissue engineering.

2-Hydroxyethyl methacrylate (HEMA) is a famous monomer due to its versatility for syntheses of polymeric biomaterials-hydrogels and hydrogel scaffolds. HEMA-based polymeric materials show favorable biocompatibility and tunable hydrophilicity. Therefore, they have multiple applications in pharmaceutical and biomedical fields such as coatings, intraocular lenses, scaffolds, and controlled drug release systems. To tune the biocompatibility, swelling, and mechanical properties of HEMA-based hydrogels, they can be combined with hydrophilic polymeric components (Prasitsilp et al. 2003; Hejcl et al. 2008; Kubinova et al. 2015; Babić and Tomić 2020; Tomić and Vuković 2020).

6.2 Design and Properties of Alginate/Gelatin/2-Hydroxyethyl Methacrylate Hydrogel Scaffolds

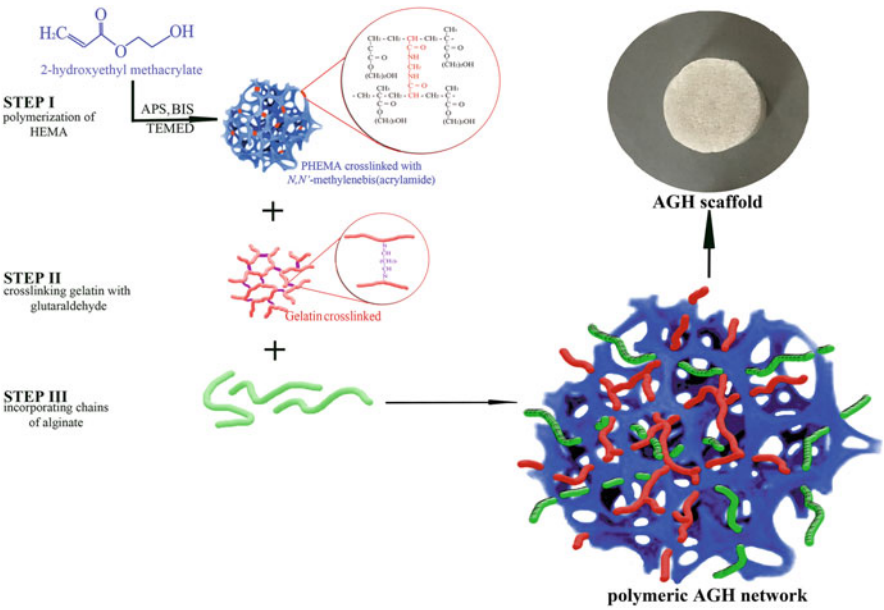
6.2.1 Alginate/Gelatin/2-Hydroxyethyl Methacrylate Polymeric Hydrogel Scaffolds

Two series of novel, porous, and degradable hydrogel scaffolds were successfully synthesized using a combination of natural polymers-alginate and gelatin, and synthetic monomer 2-hydroxyethyl methacrylate (AGH), by cryogelation and porogenation. The synthesis path is shown in Scheme 6.1.

Hydrogel scaffolds were made in three steps.In the first step, porous and the mechanically strong polymeric network was obtained by free-radical cross-linking polymerization of 2-hydroxyethyl methacrylate in the presence of an initiator, activator, and cross-linking agent. In the second step, an additional polymeric network was created by the cross-linking of gelatin. The third step is alginate chains are loaded to intertwine in the networks.

6.2.1.1 Biocompatibility of AGH Hydrogel Scaffolds

The main focus during the design of the scaffolding polymeric biomaterials which can be used in regenerative engineering and medicine is the first step related to in vitro and in vivo biocompatibility testing of the hydrogel matrices. The biocompatibility of AGH hydrogel scaffolds was evaluated in a cytotoxicity test on normal human fibroblasts (MRC5). The results of this assay for AGH samples are shown in Fig. 6.1. A50G50Hcryo, A30G70Hcryo, AHcryo, A50G50Hpor, and A30G70Hpor



Scheme 6.1 Scheme hydrogel scaffolds formation by cryogelation and porogenation

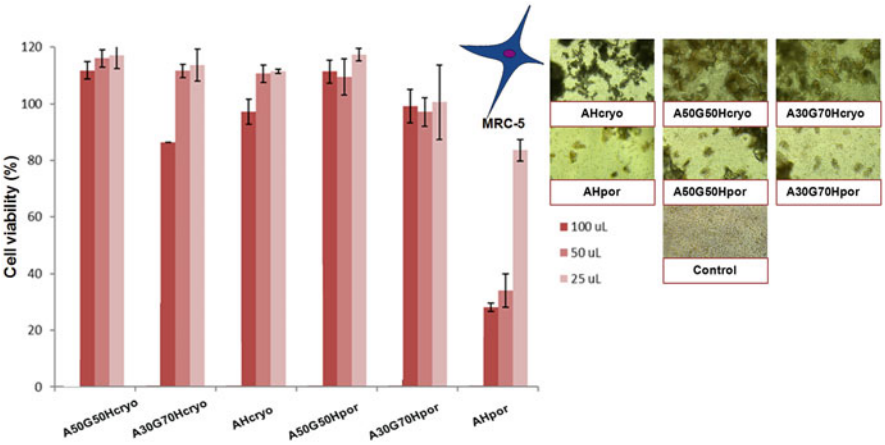


Fig. 6.1 Cell viability of AGH hydrogel scaffolds and accumulation of cells on its surface

samples showed very favorable cellular viability in contact with cell culture. The AHpor sample showed a lower level of cell survival (for higher extract concentration). It has been shown that all scaffolds, when delivered directly to cells, support the accumulation of cells on their surface (Fig. 6.1). The obtained data confirm that AGH hydrogel scaffolds demonstrate favorable level of cytocompatibility. Therefore, these scaffolding materials are suitable for applications as biomaterials in tissue regeneration.

To address whether the alginate/gelatin/methacrylate hydrogels could be used for human use, we assessed their in vivo toxicity using the zebrafish model. The zebrafish model was accepted as a valid alternative to mammalian models (rats mice) for toxicity and biocompatibility evaluation of novel biomaterial sowing to the high molecular, genetic, physiological, and immunological similarity to humans, and good correlation in response to pharmaceuticals and bioactive agents (Lieschke and Currie 2007; MacRae and Peterson 2015), simplifying thus the route to clinical trials and reducing the failure at later stages of testing (Barros et al. 2008; MacRae and Peterson 2015).

Given that the organisms at embryonic developmental stages are more sensitive to the chemical insults than as an adult, we subjected zebrafish embryos at the 6 hpf stage indirectly, to the material's extracts (embryo water in which the ground materials were extracted over 72 h at 37 °C and 180 rpm and applied as 100%, 50%, 25%, and 10% suspensions) and to the ground material (200 μg/mL) for 5 days. Obtained data showed that all AGHcryo and AGHpor scaffolding biomaterials tested, regardless of the method was no toxic to the zebrafish embryos, since there were no lethality, developmental malformation, cardiovascular disorders, or signs of hepatotoxicity (the liver necrosis and the yolk retention) (Fig. 6.2), indicating the safety of the tested materials. We showed the safety and biocompatibility (no inflammation and no immunosuppression) of 2-hydroxyethyl methacrylate hydrogels using the zebrafish model (Tomić et al. 2020). In this study, we showed

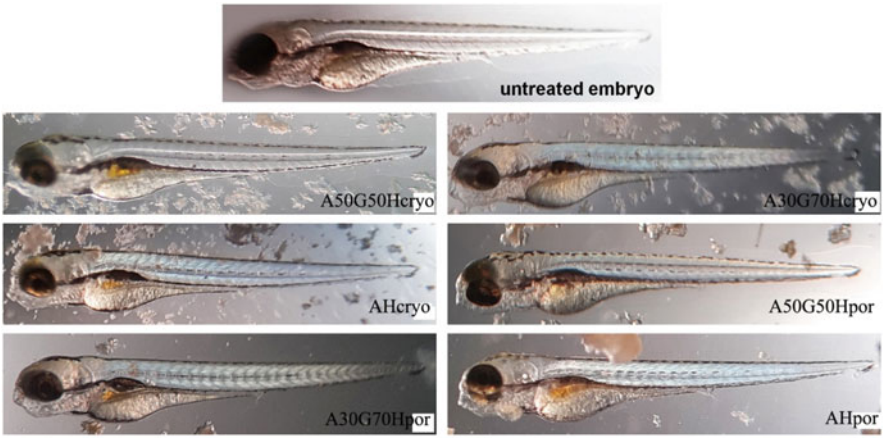


Fig. 6.2 Morphology of zebrafish embryos exposed to AGH hydrogel scaffolds (200 μg/mL) at 104 hpf

that AGH hydrogel scaffolds present also nontoxic materials, indicating their favorable potential in biomedical applications. Otherwise, the alginate-based materials have been applied as safe biomaterials for the food packing and oral delivery to zebrafish larvae or adults (Onal and Langdon 2000; Lin et al. 2016) and more recently as the carriers of drugs to improve their therapeutic outcome (controlled release, reduced toxicity, increase in efficacy) (Tzankova et al. 2017; Gao et al. 2017).

6.2.1.2 Structural Properties of AGH Hydrogel Scaffolds

Fourier transform infrared spectroscopy (FTIR) gives information about the structural characteristics of created scaffolds. FTIR spectra are tracked to find the most important bands indicating the presence of functional groups originated from scaffold components (alginate, gelatin, and 2-hydroxyethyl methacrylate). Peaks indicating the presence of HEMA in the polymeric network are on the following wavenumbers 3360 cm^{-1}, 2940 cm^{-1}, 1640 cm^{-1}, and 1710 cm^{-1} and are assigned to asymmetric and symmetric stretching vibrations (terminal hydroxyl group), methylene stretching, and terminal vinyl, and a carbonyl group. The peak at 1640 cm^{-1} can be assigned to the asymmetric stretching of COO^{-} of alginate (Wang et al. 2016). Also, the FTIR spectra show characteristic bands for alginate at 1027 cm^{-1} and 1086 cm^{-1} for its glucuronic (G) and manuronic (M) acid units. The bands at 879 cm^{-1} and 822 cm^{-1} demonstrate β-glycosidic linkages between G and M units of alginate. Besides these, characteristic bands of gelatin at 1620 cm^{-1} and 1536 cm^{-1} were observed, which correspond to amide I and amide II groups (Sharma et al. 2016). The peak at about 1150 cm^{-1}, related to C-O vibration, indicates cross-linking of gelatin, and confirms its successful incorporation into the polymeric structure. Also, the broad peak from 2900 cm^{-1} to 3600 cm^{-1} illustrates a

strong -OH bond resulting from the interaction between alginate and gelatin (Wang et al. 2016).

6.2.1.3 Water Uptake Capacity of AGH Hydrogel Scaffolds

AGH hydrogel scaffold water uptake capacity shows the hydration of dry scaffold. The AGH matrices swell up to 90% within 10 min, reaching equilibrium within 30 min. Once the scaffold was water equilibrated, further, it remains at equilibrium for a longer period. In that way shows their capacity for water uptake and the ability to retain water over a long period. AGH hydrogel scaffolds show a time-favorable hydration profile which is suitable for use in tissue regeneration.

6.2.2 Alginate/Gelatin/2-Hydroxyethyl Methacrylate Hydrogel Scaffolds Made by Cryogelation

6.2.2.1 Morphology of AGHcryo Hydrogel Scaffolds

The demands that scaffolds need to meet for proper cell attachment are to easily deliver oxygen, nutrients, water-soluble metabolites, and waste products between the inside and outside of the hydrogel scaffold. To fulfill these demands, the scaffold must exhibit a large number of interconnected pores. To examine the morphology of created scaffolds based on alginate, gelatin, and 2-hydroxyethyl methacrylate, prepared by cryogelation (AGHcryo), a scanning electron microscopy (SEM) was performed. The obtained SEM micrographs are displayed in Fig. 6.3. As can be seen, the interior of the scaffolds is highly porous, with open, interconnected pores, with spherical to elliptical shapes. The pore size was found to vary between 10 and 100 μm, which can provide efficient vascularization, proliferation, and differentiation of cells.

The surface morphology of AGHcryo scaffolds can be described as rough and porous, similar to the interior architecture of the tested scaffolds. When compared to the scaffolding materials with smooth and nonporous walls, AGHcryo scaffolds are expected to enhance the attachment of cells and their mutual interaction. The internal and surface morphology of scaffolds with different gelatin content shows different patterns that can be attributed to the more cross-links of gelatin. Obtained data for morphological testing indicate that AGHcryo scaffolds are suitable for tissue regeneration and biomedical applications.

6.2.2.2 Mechanical Properties of AGHcryo Hydrogel Scaffolds

Mechanical properties are very valuable properties of the scaffolds for tissue engineering. Therefore, the scaffold should possess good strength. To evaluate the mechanical properties of hydrogel scaffolds based on alginate, gelatin, and 2-hydroxyethyl methacrylate, prepared by cryogelation (AGHcryo), Young's modulus (E) was determined. The results revealed E values of AGHcryo samples in the range of 1.251–9.755 MPa, thus clearly indicating that scaffold composition determines their mechanical properties. The lowest E value exhibited the AHcryo sample (1.251 MPa), while A50G50Hcryo showed the highest value (9.755 MPa).

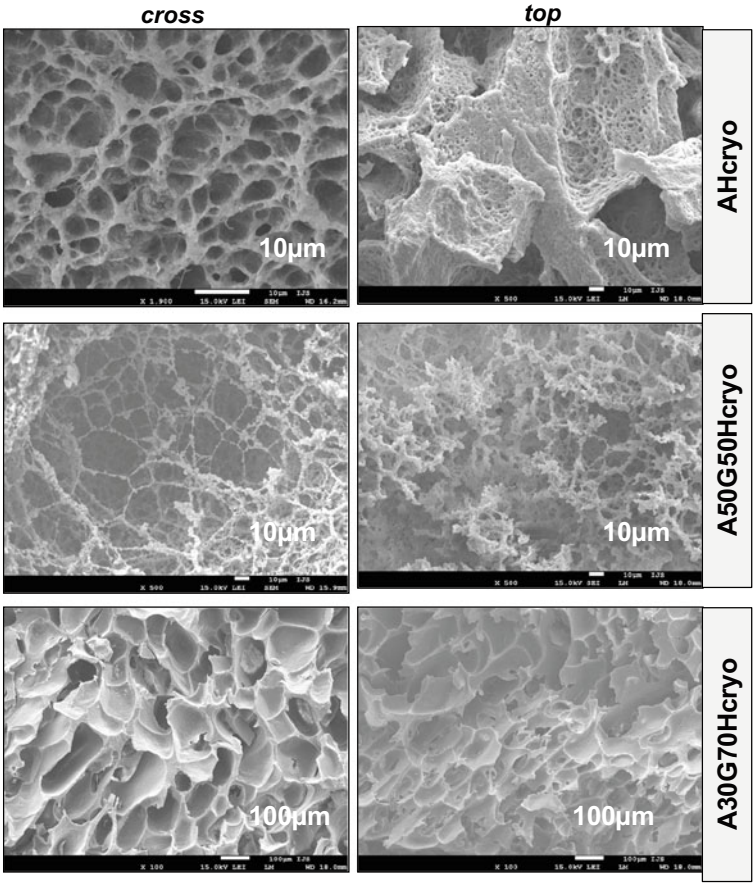


Fig. 6.3 SEM micrographs of AGHcryo hydrogels

The presence of gelatin resulted in a more compact, mechanically stronger structure, but with the further increase of gelatin content value of Young's modulus was decreased. Certainly, according to known data related to the mechanical characteristics of native tissues, AGHcryo scaffolds possess great potential for tissue regeneration application, especially for soft tissues and skin. The desired mechanical strength can be adjusted by synthesis optimization and adjusting the component ratios.

6.2.2.3 Porosity of AGHcryo Hydrogel Scaffolds

The porosity of hydrogel scaffolds based on alginate, gelatin, and 2-hydroxyethyl methacrylate, prepared by cryogelation (AGHcryo) was evaluated by the bulk density method. The porosity values of hydrogel scaffolds depend on the composition of scaffolds. The results obtained reveal a high level of porosity of all tested samples, over 80%. The highest porosity showed the AHcryo sample (84.63%),

while the lowest value has an A50G50Hcryo sample (82.26%). The presence of gelatin slightly decreased the porosity, most probably due to the more cross-links of gelatin. Results indicate that the porosity of the AGHcryo hydrogel scaffolds obtained by cryogelation can be adjusted and adapted by modifications of scaffold composition and variations of components ratio. Generally, there is no change in porosity due to the increased surface area and hydrophilicity of gelatin and alginate.

6.2.2.4 Hydrophilicity of AGHcryo Hydrogel Scaffolds

The surface hydrophilicity of the alginate/gelatin/2-hydroxyethyl methacrylate hydrogel scaffolds, prepared by cryogelation was assessed by the contact angle measuring through droplet water spread on a surface. As it is well known, the hydrophilicity of material is reflected in contact angle low values. The results obtained from the static water contact angle are presented in Fig. 6.4. A50G50Hcryo hydrogel scaffold was found to be fully hydrophilic, due to the completely wet surface and disappearance of the water drop. The contact angle values for A30G70Hcryo and AHcryo samples are 81.55 and 81.20. The surface hydrophilicity of AGHcryo scaffolds certainly depends on the composition and ratio of natural polymers used for the syntheses. Obtained data showed favorable hydrophilicity of

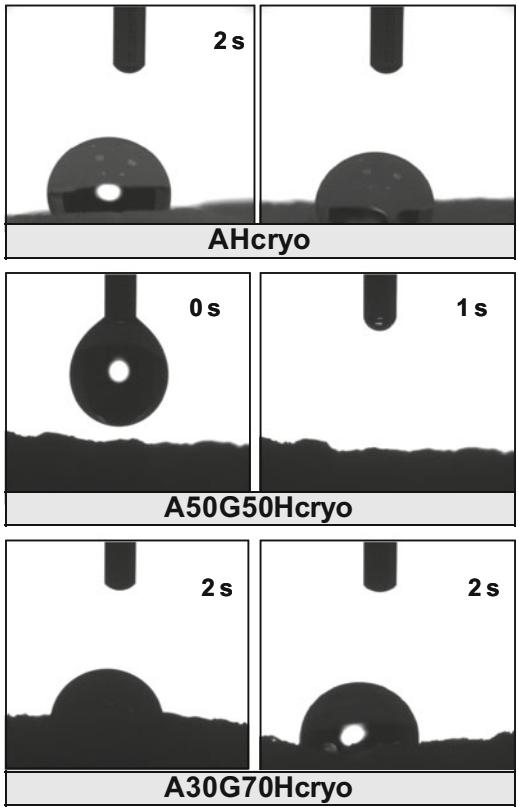


Fig. 6.4 The water contact angle of AGHcryo hydrogel scaffolds

AGHcryo hydrogel scaffolds. Thus the hydrophilic character of hydrogels based on alginate, gelatin, and 2-hydroxyethyl methacrylate, prepared by cryogelation can provide efficient attachment, proliferation, and further growth of the cells.

6.2.2.5 In Vitro Degradation Properties of AGHcryo Hydrogel Scaffolds

The in vitro degradation process was monitored in controlled, physiologically conditions-simulated buffer fluid of pH 7.40 and at a temperature of 37 °C. The weight loss, as an indicator of degradation of alginate/gelatin/2-hydroxyethyl methacrylate hydrogel scaffolds, prepared using cryogelation was investigated for 3 months. The tested scaffolds degraded up to 11% after 3 months. The highest weight loss was detected for the AHcryo scaffold (11.33%), while the lowest weight loss exhibited A30G70Hpor (3.41%). The decrease of alginate content and the increase of gelatin in scaffolds resulted in decreased biodegradability. As it is assumed, the degree of cross-linking of gelatin has a prominent influence on the scaffolds biodegradability.

6.2.3 Alginate/Gelatin/2-Hydroxyethyl Methacrylate Hydrogel Scaffolds Made by Porogenation

6.2.3.1 Morphology of AGHpor Hydrogel Scaffolds

Hydrogel scaffolds designed for tissue regeneration must be highly porous with an open interconnected pore, to permit a large surface area relative to the scaffold's volume. The porosity is directly coherent to the function of the scaffold. Pronounced and interconnected porosity will promote cell growth, uniform cell distribution, and assist the neovascularization of the matrix. According to the latest studies, scaffolds should have a distribution of pores ranging from micrometers to 100 μm, improving the capillarity of the scaffolds and enabling the ingrowth of cells (Kim et al. 2019). Morphological properties of the alginate/gelatin/2-hydroxyethyl methacrylate hydrogel scaffolds, prepared by porogenation (AGHpor) were tested using scanning electron microscopy (SEM) (Fig. 6.5). SEM micrographs of the cross-sections revealed a uniform porous structure with interconnected pores. No phase separation and occurrence of crystalline regions phenomena were detected, which indicate the compactness and compatibility between components of the resulting polymeric network scaffolds. The addition and increase of gelatin content in the scaffolds reflected in a more porous, almost like a honeycomb, structure with the smaller pores finely distributed in larger pores. Scaffold's architecture can be depicted as the interior is porous, with open, interconnected pores, with spherical to elliptical shapes, thus providing efficient nutrient flow and distribution, the proper vascularization, proliferation, and differentiation support for the cells.

Also, micrographs of the scaffold's surfaces revealed a range of pore sizes from a few microns to 200 μm, as well as their interconnections. The larger ones are filled with small pores, which indicate that the surface porosity can contribute to the interactions between these scaffolding materials and the cells, which implies their faster attachment.

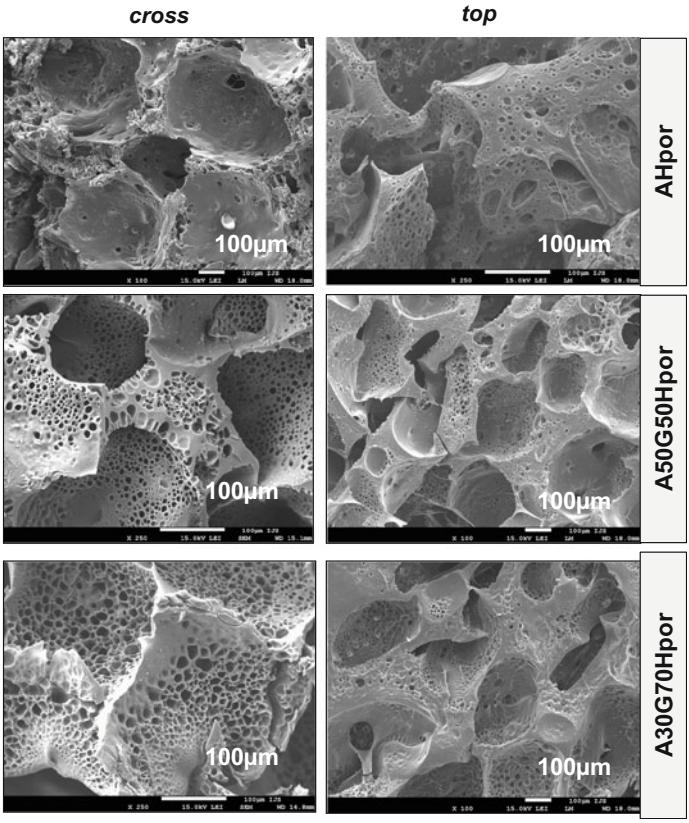


Fig. 6.5 SEM micrographs of AGHpor hydrogel scaffolds

6.2.3.2 Mechanical Properties of AGHpor Hydrogel Scaffolds

Mechanical properties are a very valuable parameter for scaffolding engineering because scaffolding must withstand handling and should show initial load-bearing capacity. To achieve this, the challenge is to combine hydrogel scaffolding of natural/synthetic origin. Strength tests were performed to assess the mechanical performance of the scaffolds. The focus of current researches in tissue engineering is to make a porous scaffold that mimics the native extracellular matrix (ECM). Among the demands, mechanical properties are essential factors to consider in scaffolds production. Mechanical strength is defined by the impact resistance of final products to maintain the integrity of the scaffold during implantation (Tran et al. 2018). The material application for tissue engineering requires the resemblance of deformable characteristics between the biomaterial and the analog tissue. The cell attachment, growth, and function in such a 3D structure depend on the scaffold mechanical properties (Lim et al. 2019). Elastic modulus, as a representative of the stiffness of the biomaterial, is an indicator of similarity between the biomaterial aimed to replace or repair particular tissue.

Young's modulus (E) of hydrogel alginate/gelatin/2-hydroxyethyl methacrylate scaffolds, prepared by porogenation was determined and the obtained values depend on the composition of the scaffolds. As was expected, the reverse trend of calculated E values was noticed compared to the determined porosity of the hydrogel scaffolds. The lowest E value exhibited A50G50Hpor (0.988 MPa), the scaffold with the highest level of porosity. The increase of gelatin content resulted in a more compact, mechanically stronger structure, thus the A30G70Hpor sample showed the highest value of Young's modulus (4.056 MPa). AHpor scaffold has E value near the highest calculated (3.102 MPa).

The obtained data indicate that the mechanical properties can be tuned through control of the scaffold's composition. According to known data, the native skin has Young's modulus in a range of 4.6–20.0 MPa, while the E value of cartilage is 12.0 MPa (Joodaki and Panzer 2018; Żak et al. 2011). Thus, alginate/gelatin/2-hydroxyethyl methacrylate scaffolds, prepared by porogenation can realize the requirements for the specific biomedical application. Additionally, by altering scaffold composition and the ratio of components, favorable mechanical strength could be reached.

6.2.3.3 Porosity of AGHpor hydrogel Scaffolds

The specifically defined porosity of the scaffolding materials is certainly one of the crucial requests that must be fulfilled to create an adequate 3D matrix for cell attachment, viability, and growth to successfully regenerate damaged tissue. The level of porosity of a biomaterial can be evaluated by the bulk density method, as it was performed in this study. The results obtained for AGHpor hydrogel scaffolds show that AGHpor tested scaffolds exhibited values of porosity in the range of 28–70%, thus confirming the results of the morphological study. The presence of gelatin increased the porosity of the hydrogels. The minimum value showed AHpor (28.48%), due to the intertwined chains of alginate in the polymeric matrix which decreases available free space. The highest porosity exhibited for the A50G50Hpor sample (69.92%). Further increase of gelatin content slightly decreased the porosity of the hydrogel scaffolds, probably due to the more cross-links of gelatin. Thus, scaffold composition and component variations play a notable role in tailoring the specific and targeted porosity of these biomaterials.

6.2.3.4 Hydrophilicity of AGHpor Hydrogel Scaffolds

The extracellular matrix hydrophilicity is a valuable parameter affecting cell adhesion processes in tissue engineering. Low surface wettability influences the attachment, colonization, and growth of cells (Cho et al. 2015). Focal adhesion represents the interaction between cells and scaffold or the specific sites of their contacts. When the scaffold surface has a hydrophobic character, it disables the focal adhesion. Nowadays, the development of scaffolding materials is focused on surface modifications to achieve desirable hydrophilicity (Chang and Wang 2011).

The surface hydrophilicity of hydrogel scaffolds is assessed by measuring the contact angle through the water droplet spread on a surface. A lower contact angle indicates higher surface hydrophilicity. Figure 6.6 shows the static water contact

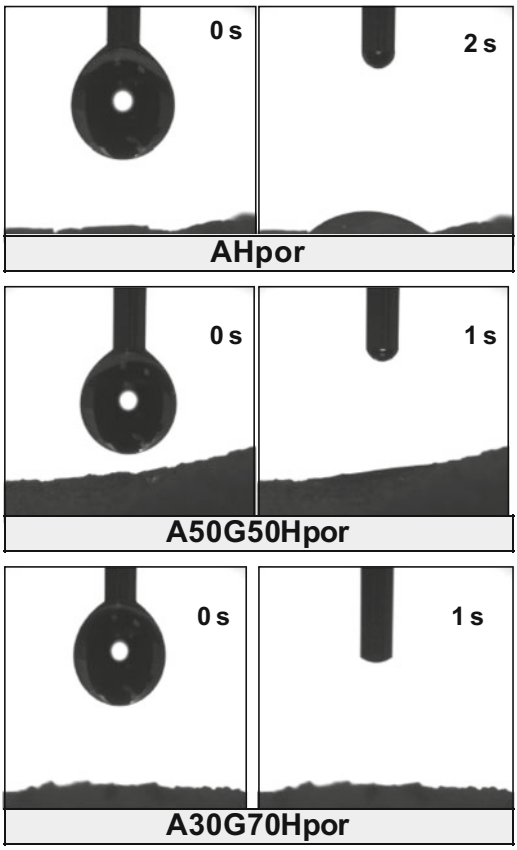


Fig. 6.6 The water contact angle of AGHpor hydrogels

angle results obtained reveal the hydrophilic character of AGHpor hydrogel scaffolds. As can be observed, A50G50Hpor and A30G70Hpor hydrogel scaffolds are completely hydrophilic, where water has completely wetted their surface, with the drop disappearing immediately after being placed on the surface of the hydrogel. AHpor sample shows a slightly small wetting angle. The obtained results suggest that the AGHpor hydrogel scaffolds are suitable as scaffolding biomaterials to enable favorable adhesion, proliferation, and differentiation of various types of cells.

6.2.3.5 In Vitro Degradation Properties of AGHpor Hydrogel Scaffolds

Scaffold degradation and the matching rate between scaffold degradation and new tissue growth are crucial factors for advanced tissue regeneration (Zhang et al. 2014). Synthetic biodegradable polymers can be used extensively for scaffold fabrication due to desirable mechanical support to the 3D matrix, and the possibility to control the degradation (Sung et al. 2004). Natural polymers, biopolymers, e.g., alginate and gelatin, are suitable candidates for porous ECM-mimicking scaffolds, owing to their biodegradable and biocompatible nature (Bettinger et al. 2007).

The weight loss, as an indicator of degradation, for alginate/gelatin/2-hydroxyethyl methacrylate hydrogel scaffolds is investigated during 3 months, in simulated buffer fluid of pH 7.40, at 37 °C. The scaffolds are degraded up to 3% after 3 months. The highest weight loss exhibited A30G70Hpor (3.07%), suggesting that the increase of gelatin content contributed to the biodegradability of the hydrogels. AHpor and A50G50Hpor degraded at a similar rate, with weight loss of around 1.60%.

From the obtained data for the rate of weight loss of AGHpor scaffolding biomaterials, it can be said that the composition of the scaffolds affects the degradation process in such a way that the desired degradation rate can be achieved by varying and fine adjustment of the component ratio.

6.3 Summary and Future Perspectives

In our researches, two series of scaffolding biomaterials, based on alginate/gelatin/methacrylate, were synthesized by free-radical polymerization/cross-linking reaction using special methods of cryogelation and porogenaion. All samples showed favorable properties thus suitable for tissue regeneration and engineering-biocompatibility, water uptake, morphology, mechanical properties, porosity, hydrophilicity, and degradation.

6.3.1 AGHcryo Hydrogel Scaffolds

We have successfully developed hydrogel scaffolds of alginate, gelatin, and 2-hydroxyethyl methacrylate by free-radical cross-linking polymerization using cryogelation. Results obtained for cytotoxicity, it can see that AGHcryo hydrogel scaffolds do not show significant changes in cell viability with the composition scaffold, and are exceptionally cytocompatible, and therefore suitable as polymeric biomaterials for tissue regeneration. The in vivo zebrafish embryos assay test showed that the AGHcryo scaffolding materials tested were no toxic effects, since there was no lethality, developmental malformation, cardiovascular disorders, or signs of hepatotoxicity, which means they are safe to use in tissue regeneration. The spectroscopic characteristics of the AGHcryo scaffolding materials demonstrated the presence of bands indicating that the polymeric networks were constructed of alginate, gelatin, and 2-hydroxyethyl methacrylate components. Water uptake behavior for AGHcryo hydrogel scaffolds demonstrated a time-favorable hydration profile which is suitable for use in tissue regeneration. The morphology of AGHcryo hydrogel scaffolds is highly porous, with open, spherical to elliptical shapes, interconnected pores, with pore size in the range of 10–100 μm, which can provide efficient vascularization, proliferation, and differentiation of cells that meet the requirements to be used for tissue regeneration. The mechanical properties of AGHcryo scaffold samples, represented by the modulus of elasticity, are in the range of 1.251–9.755 MPa, and depend on the composition of the scaffolds. These

values indicate the great potential of AGHcryo scaffolding materials for tissue regeneration, especially for soft tissues and skin. The porosity data of AGHcryo scaffold samples are in the range of 80–85%. This parameter can be adjusted and adapted by modifications of scaffold composition and variations of components ratio. A50G50Hcryo hydrogel scaffold was found to be fully hydrophilic. The contact angle values for A30G70Hcryo and AHcryo samples are 81.55 and 81.20. Obtained data showed favorable hydrophilicity of AGHcryo hydrogel scaffolds. Thus the hydrophilic character of alginate/gelatin/2-hydroxyethyl methacrylate hydrogels, prepared by cryogelation can provide efficient cell attachment, proliferation, and further growth. The in vitro degradation process was monitored for 3 months. The tested AGHcryo hydrogel scaffolds degraded up to 11% after 3 months. The highest weight loss was detected for the AHcryo scaffold, while the lowest weight loss exhibited A30G70Hpor.

6.3.2 AGHpor Hydrogel Scaffolds

We have designed alginate/gelatin/2-hydroxyethyl methacrylate hydrogel scaffolds by free-radical polymerization/cross-linking using porogenation. The cytotoxicity assay for AGHpor hydrogel scaffolds shows satisfied cell viability (only AHpor for higher extract concentration shows lower cell viability). In vivo zebrafish embryos assay test showed that the AGHpor scaffolding materials were no toxic effects since there was no lethality, developmental malformation, cardiovascular disorders, or signs of hepatotoxicity. Therefore, AGHpor scaffolding materials are safe to use in tissue regeneration applications as polymeric biomaterials. The spectroscopic characteristics of the AGHpor scaffolding materials demonstrated the presence of bands indicating that the polymeric networks were built of alginate, gelatin, and 2-hydroxyethyl methacrylate components. Water uptake behavior for AGHpor hydrogel scaffolds displayed a time-favorable hydration profile which is suitable for use in tissue regeneration. Morphology of AGHpor hydrogel scaffolds indicates highly porous structures, with open, spherical to elliptical shapes, interconnected pores. Pore size is in the range of 10–100 μm. This morphology pattern can provide efficient vascularization, proliferation, and differentiation of cells, which are prerequisites for tissue regeneration. The modulus of elasticity of AGHpor scaffolds is in the range of 0.988–4.056 MPa and depends on the scaffold composition. These values indicate the good potential of AGHpor hydrogel scaffolds for tissue regeneration, particularly for soft tissues. The porosity data of AGHpor scaffold samples is in the range of 28–70%. This feature can be tuned and adapted by modifications of scaffold composition and variations of components ratio. A50G50Hpor and A30G70Hpor hydrogel scaffolds were found to be fully hydrophilic. AHpor sample showed a slightly small wetting angle. The obtained results suggest that AGHpor hydrogels are favorable scaffolding biomaterials for cell adhesion, proliferation, and differentiation. The tested AGHpor hydrogel scaffolds degraded up to 3% after 3 months. The highest weight loss exhibited A30G70Hpor (3.07%), suggesting that the increase of gelatin content contributed to the biodegradability of the

hydrogels. AHpor and A50G50Hpor degraded at a similar rate, with weight loss of around 1.60%.

As the most important achievements of these extensive researches, it can be stated that AGH hydrogel scaffolds created by cryogelation showed slightly more favorable properties for tissue regeneration compared to AGH hydrogel scaffolds created by porogenation. Another important fact is that more advantageous properties for tissue regeneration are achieved when the polymeric chains of alginate are intermingled into polymeric networks of gelatin and 2-hydroxyethyl methacrylate than into a polymeric network of 2-hydroxyethyl methacrylate. Thanks to the significant progress already made, along with others that will be made in the near future. The safe and effective clinical application of alginate/gelatin/methacrylate polymeric scaffolding systems is expected to accelerate and expand soon.

Hydrogel scaffolding materials have a significant role in regenerative medicine and tissue engineering. The future advances and success of hydrogel scaffolds are based on the new classes of polymers syntheses or the modification of natural polymers to solve certain biological and medical obstacles. Most scientific research shows that hydrogel scaffolding studies have a great future. New aspects to hydrogel scaffold design are increased in the investigation of these biomaterials, with an emphasis on rapid response, self-assembly, high and good mechanical properties, and super-porosity.

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Tissue Engineering Applications of Marine-Based Materials

7

Hurriyet Polat, Nuket Zeybek, and Mehmet Polat

Abstract

Tissue engineering is a promising approach in replacing or improving tissues lost or has become nonviable due to disease or trauma by the use of scaffold materials by combining engineering and biochemical/physicochemical methods. Its purpose is to create suitable matrices that support cell differentiation and proliferation toward the formation of new and functional tissue.

Marine-based natural compounds are potential scaffold feedstock material in tissue engineering owing to their biocompatibility and biodegradability while providing excellent biochemical/physicochemical properties. Numerous application areas and various fabrication routes techniques described in the literature attest to the importance of these materials in tissue regeneration. This review has been carried to merge the information from a large number of studies on the marine-based scaffold materials in tissue engineering into a coherent summary.

Keywords

Marine · Biomaterial(s) · Tissue engineering · Scaffold(s)

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7.1 Introduction

7.1.1 Tissue Engineering

When tissues or organs have been seriously diseased or lost as a result of disease or trauma, artificial transplantation may need to be employed to reconstruct damaged structures (Mandrycky et al. 2017) using tissues from the patient, another person, or different species (Ikada 2006). Although life-saving, the process needs to overcome the difficulties such as anatomical limitations presented by autografts, shortage of organ donations, rejection by the immune system, donor site morbidity, and infection risks.

Tissue engineering aims to create suitable matrices called scaffolds that support cell differentiation and proliferation toward the formation of new and functional tissue. It takes advantage of engineering methods for the formation of new viable biological substitute tissues to restore, maintain, or improve functionality while overcoming tissue biocompatibility and rejection problems (Atala 2004). The interplay among the cells, biological factors (cell signals which initiate growth or growth factors and signal pathways or extracellular matrix) and the scaffold material is called

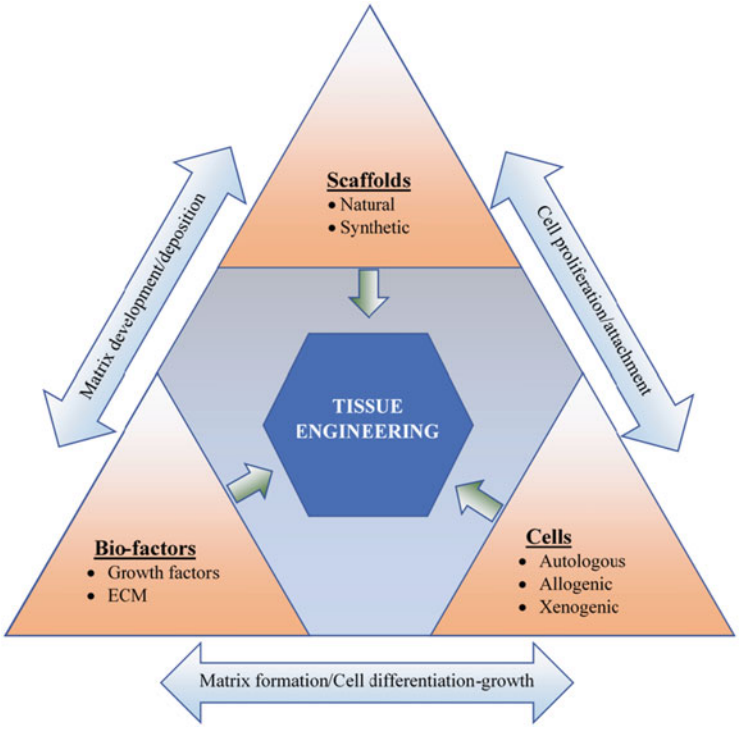

Fig. 7.1 Tissue engineering triad

the tissue engineering triad and determines the outcome of any tissue engineering attempt (Fig. 7.1) (Jagur-Grodzinski 2010; Eltom et al. 2019; Celikkin et al. 2017).

Cells of biological tissues, which are the primary ingredients of any tissue engineering attempt (Briquez and Hubbell 2020), can be classified into three categories: autologous (patient's own), allogeneic (human other than the patient), and xenogeneic (animal origin). Autologous cells are most suitable for transplantation since allogeneic and xenogenic cells may pose infection risks and immunogenic reactions (Ikada 2006; Fishman et al. 2012). Differentiated cells of the same tissue type, stem cells, and some other cell types such as dermal cells, may be preferred depending on the specifics of the application. The cells selected must be co-cultured to encourage tissue level variability and should withstand the mechanical stresses when inserted into the body.

Cells do not divide under normal conditions unless they are stimulated by signals from other cells. Signaling among the cells is achieved by diffusible ligands (in the form of naturally secreted cytokines or steroid hormones), which bind to their receptors on the surface of the target cells. These signal-transducing molecules, called the growth factors, are responsible for initiating and promoting cell proliferation, differentiation, and maturation. The concentration of the growth factor affects the response of a target cell to a specific growth factor. Extracellular matrix (ECM) is a network of noncellular macromolecules such as collagen, enzymes, and glycoproteins and acts as a signaling pathway and providing structural support to surrounding cells. ECM is generated during the development of any tissue or organ and is responsible for regulating almost all of the cellular functions. It is mainly composed of water, proteins, and polysaccharides though its composition and topology are unique for each tissue. ECM components and the cell adhesion receptors interact with each other forming a complex network in which cells reside in all tissues and organs (Humphries et al. 2006; Leitinger and Hohenester 2007; Harburger and Calderwood 2009; Xian et al. 2010; Frantz et al. 2010). Besides providing essential physical scaffolding for attachment for the cellular constituents, ECM also initiates crucial biochemical and biomechanical cues that are required for tissue morphogenesis, differentiation, and homeostasis.

7.1.2 Scaffold Design

In tissue engineering, a synthetic three-dimensional scaffold made up of either man-made or natural materials is seeded with suitable living cells and growth factors. The cells secrete their ECM and provide a framework within the scaffold for cell proliferation, differentiation, and attachment (Briquez and Hubbell 2020; Howard et al. 2008). Regardless of the tissue type, three main factors influence scaffold design and manufacturing: i) biological factors, ii) structural factors, and iii) chemical and physicochemical properties of scaffold surfaces (O'brien 2011; Hutmacher 2000; O'brien 2011; Loh and Choong 2013; Declercq et al. 2014; Velasco et al. 2015). These factors that form another triad for scaffold design (Fig. 7.2) are discussed below in more detail.

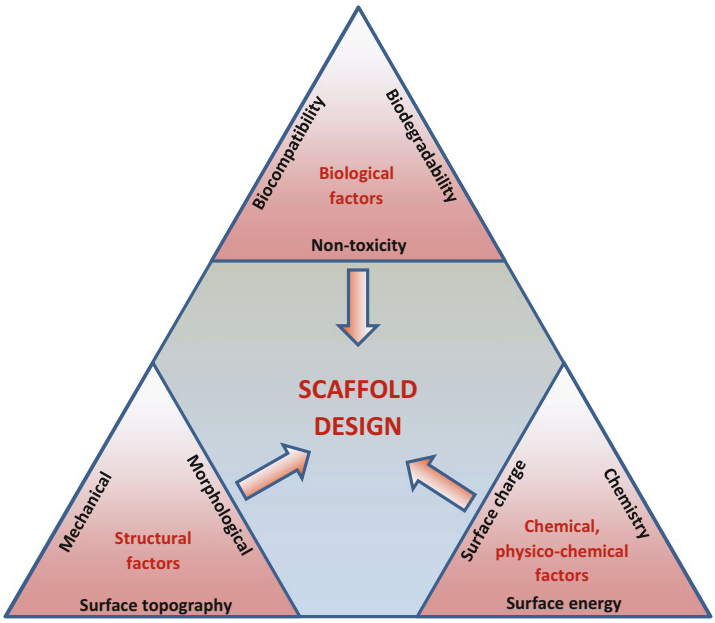

Fig. 7.2 Scaffold design triad

Biological factors: The biological factors can be broadly grouped under biocompatibility and biodegradability. Biocompatibility that defines the proper microenvironment for the cells to adhere, multiply, migrate, and function normally to create new tissue within a scaffold (Naahidi et al. 2017; Kohane and Langer 2010) is the foremost criterion of scaffold manufacturing. Biocompatibility does not presume an absolutely inert matrix, which may lead to poor vascularization and deceleration of regeneration (Ivanov et al. 2019). It has been demonstrated that the presence of moderate inflammatory reactions has a stimulating impact on cell settlement and vascularization (Santos et al. 2013; Spiller et al. 2014; Crupi et al. 2015).

Biodegradability is another important factor since scaffolds are not aimed to be permanent structures; they should degrade gradually and be replaced as the cells produce their ECM. matrix. By-products of biodegradation should not be toxic and harm other organs. Since an inflammatory response caused by macrophages is required for degradation, the field of immunology is important in tissue engineering (Lyons et al. 2010; Asghari et al. 2017; Liu and Czernuszka 2007).

Structural factors: A scaffold should possess certain physical characteristics, which can be grouped under mechanical and structural properties. Mechanical properties such as strength, elasticity, and durability, which define the limits for the scaffold for maintaining its integrity during implantation or use and for mimicking the anatomical structure and behavior of the native implantation region, are very important considerations in scaffold design. Some of the common parameters

employed in quantifying mechanical properties are Young's modulus, compressive/tensile strengths, and breaking strain (elongation at break) values.

Young's modulus or the modulus of elasticity, which is the ratio of stress (the compressive or tensile force applied per unit area) to strain (the relative deformation of the material resulting from the applied stress) measures a material's resistance to being deformed elastically under stress. Stiff materials resist changing their shape under elastic loads and have high Young's moduli, whereas flexible materials that deform easily have low Young's modulus. For example, Young's moduli of various tissues are 0.5-1 kPa for fat, 20-40 kPa for intestine, 1-1.5 MPa for cartilage, and 5 KPa-140 MPa for skin and 15-20 MPa for bone (Zahouani et al. 2009; Liu et al. 2015a; Handorf et al. 2015; Kalra et al. 2016; Ceccaldi et al. 2017). Note that the same biological material such as skin may display a large variation in Young's modulus due to anisotropy.

The strength of the material measures the amount of stress it can withstand before it fails. Some example compression strength values are 2-12 MPa for cancellous (spongy) bone and 5 to 30 MPa for skin (Boccardi et al. 2015; Tran et al. 2018; Prasadh and Wong 2018). Breaking strain (or elongation at break or fracture strain) is the ratio between final and initial lengths at the breaking point. Some example breaking strain values are 10% for human myocardium, 10% for human cardiac cells, 60% for human cornea, and 35–115% for native skin depending on the direction (Tran et al. 2018; Ozcelik et al. 2013; Kalishwaralal et al. 2018).

Porosity and pore interconnectivity are important morphological properties and allow the diffusion of nutrients and oxygen to cells and removal of waste products are critical for tissue vascularization and new tissue formation (Declercq et al. 2014; Hollister 2005; Causa et al. 2007; Loh and Choong 2013; Kang and Chang 2018). Surface topography regulates cell adhesion, migration, proliferation, differentiation, and ECM secretion and plays an active role in cell and tissue organization along with scaffold-cell interactions (Miyoshi and Adachi 2014; Mansouri and Bagheri 2016; Xiong et al. 2020).

Chemical and physicochemical properties: Cell attachment, migration, proliferation, and contraction proceed as a result of the signaling among the cells, which is achieved by diffusible ligands (the growth factors), which bind to their receptors on the surface of target cells. Therefore, the chemical composition of the scaffold, which defines the molecules displayed on its surface, will determine the eventual success of the scaffold design. Harley and Yannas (2014) report that several synthetic polymers, which cannot express the necessary ligands on their surface, have not induced regeneration and the scaffold surface may need to be modified chemically to display appropriate ligands in these cases.

Cells do not directly interact with the surface, but rather with the surface-attached proteins through direct binding to receptors within the cellular membrane, whose charges are predominantly negative due to the presence of phospholipids, proteins, and polysaccharide conjugates (Bondar et al. 2012; Wei et al. 2014; Schulz et al. 2018). Hence, wettability (i.e., surface energy) and surface charge are the two most critical parameters in initiating and maintaining cell proliferation by favoring interactions between the scaffold surface molecules and the cells and ECM proteins

(Bartis and Pongrácz 2011; Katti et al. 2008; Schaap-Oziemlak et al. 2014; Dhowre et al. 2015). It has been reported that while an increased surface charge promotes cell attachment (de Rosa et al. 2004; Schneider et al. 2004; Chang et al. 2016; Tan et al. 2017; Schulz et al. 2018), positively charged surfaces induce differentiation processes of stem cells (Liu et al. 2010; Tan et al. 2012; Zhang et al. 2015; de Luca et al. 2016). The charge in some common marine-based scaffold materials may show significant variations. For example, chitosan is positively charged up to a pH of 7, whereas alginate is negatively charged at all pH values (Zeeb et al. 2015; Schulz et al. 2018). Considering that the magnitude and sign of this charge can be regulated by modulating pH or ionic strength of the medium to some extent, it is clear that a good understanding of the surface physicochemical properties offers various avenues for scaffold design.

7.1.3 Scaffold Materials Frequently Used in Tissue Engineering

An ideal scaffold is expected to mimic the biomechanical function, and topological and microstructural characteristics of the native ECM . This requires some essential features such as high surface/volume ratio, high porosity, pore interconnection (to support cell tissue penetration), suitable pore size, proper morphology, and proper surface physicochemical properties. Therefore, material selection should be carried out very carefully.

Human-originated biomaterials created by the elimination of cellular and nuclear materials from natural tissues or organs stand out as the first choice. Since they are rich in growth factors, they can imitate critical aspects of the natural ECM and show excellent biocompatibility (Tiitu et al. 2008). However, the sources of this type of biomaterials are limited. Therefore, a wide range of natural or synthetic polymers and ceramic materials have been considered as alternative biomaterials (Yang et al. 2001, 2002; Ma 2004; Singh et al. 2016; Ahmed et al. 2018; Abbasian et al. 2019; Nikolova and Chavali 2019). Natural materials can be grouped into polysaccharides and protein origin and microbially fermented (Salerno and Pascual 2015). The synthetic materials can be divided into polymeric- and ceramic-based biomaterials (Hutmacher et al. 2001; Willerth and Sakiyama-Elbert 2019). The listing of specific materials under these groups is presented in Table 7.1 with some most recent related studies. Recent papers by O'brien (2011), Garg et al. (2015), Nikolova and Chavali (2019), Qu et al. (2019), Shick et al. (2019), and Williams (2019) provide extensive reviews of the natural or synthetic scaffold materials, their fabrication methods, and applications.

Synthetic polymers and ceramics, which have longer shelf lives, can be produced on larger scales and can be tailored to have controllable mechanical, chemical, and physicochemical properties during synthesis. However, these materials have some disadvantages that limit their applications in the biomedical field such as limited biocompatibility, biodegradability, lack of biologically active sites for cell viability and growth, and inconsistency in composition. In addition, the degradation by-products of some synthetic polymers can negatively affect the surrounding

Table 7.1 Classification of scaffold raw biomaterials

Type	Origin	Examples	Ref
Natural biomaterials	Polysaccharide origin	Agar/agaroseAlginate Carrageenan Chitin/chitosan Hyaluronan Fucoidan	Feng et al. (2020), Zhou et al. (2018), Bagheri et al. (2019), Felfel et al. (2019), Kozlowska et al. (2019), Balagangadharan et al. (2018), Zhuo et al. (2017), Schwab et al. (2020)
	Protein origin	Collagen Gelatin Fibrin Silk	Govindharaj et al. (2019), Fernández-Cervantes et al. (2020), Coelho et al. (2017), Ali et al. (2020), Chawla et al. 2020), Luetchford et al. (2020), Bonhome-Espinosa et al. (2020), Deepthi and Jayakumar (2018), Du et al. (2018), Zhang et al. (2017a)
	Microbial fermented	Polyhydroxyalkanoates	Zhao et al. (2003), You et al. (2011)
Synthetic biomaterials	Polymer biomaterials	Poly(dioxanone) Poly(caprolactone) Poly(glycolic acid) Poly(lactic acid) Poly(butylene succinate)	Cheng and Chen (2017), Kalitheertha Thevar et al. (2019), Chen et al. (2020a), Mironov et al. (2019), Chuan et al. (2020), Shirazi et al. 2016), Huang et al. (2018)
	Ceramic biomaterials	Alumina Bioactive glass Hydroxyapatite Calcium phosphate	Abbasian et al. (2019), Mondal et al. (2020), Milovac et al. (2014), Kolan et al. (2017), Pandey et al. (2020)

tissues and cause inflammatory reactions (Asti and Gioglio 2014; Abbasian et al. 2019).

Natural-based polymers (biomaterials) offer an advantage similar to biological macromolecules, where the biological environment is prepared to recognize and to deal with metabolically. Owing to their similarity with the extracellular matrix, natural polymers may also avoid the stimulation of chronic inflammation or immunological reactions and toxicity, often detected with synthetic polymers (O'brien 2011; Silva et al. 2012). These materials are also biodegradable, allowing host cells to produce their own extracellular matrix in time and replace the degraded scaffold. Therefore, they are promising candidate materials for tissue engineering applications owing to these biophysical and biochemical properties that can induce the correct biological response for both in vitro and in vivo tests. In addition, the environmental impact of these materials, both during production and application, is minimal, which leads to decreased plastic waste formation and carbon dioxide emissions (Salerno and Pascual 2015; Hutmacher et al. 2001). Despite these advantages, the problems encountered in manufacturing consistent (homogeneous and reproducible) structures and obtaining satisfactory mechanical strengths and durabilities are the challenges, which must be overcome for these materials to find a wide application area in

scaffold engineering (Märtson et al. 1999; Zhou et al. 2013; Wang and Drzal 2012; Rao et al. 2018; Ullah and Chen 2020). As a result, the use of composite scaffolds comprised of different phases is becoming increasingly common (Jayakumar et al. 2011; Kumar et al. 2011; Sheik et al. 2018; Zeimaran et al. 2017; O'brien 2011).

7.2 Biomaterials of Marine Origin

Nature presents a huge potential for technological breakthroughs through two fundamental ways: Firstly, it provides wonderful examples of function and compatibility precipitated through millions of years of trial and error for solving the intricate problems encountered in designing functional products basically. There are numerous examples of inspired biomimicry from the very large (fins of wind turbines after humpback whales or noses of bullet trains after kingfisher birds) to the very small (swimsuits after shark skins or self-cleaning surfaces after leaves of lotus plant). Secondly, the nature offers a plethora of natural, ready-made compounds from simple molecules to very complicated molecular assemblies in the form of proteins, polymers, and inorganic matrices or composites.

The oceans, which comprise about 70% of the earth's surface and 90–95% of the biosphere, offer a tremendous source of a broad range of economical, biocompatible, and biodegradable natural materials for potential use in biotechnological applications owing to their immense volume and biodiversity. The skins, scales, fins, and bones of marine animals, as well as sponges and seaweeds, are feedstocks for an enormous variety of biomaterials ranging from proteins (collagen) and polysaccharides (alginate, carrageenan, gelatin, fucoidan, and chitin) to bioceramics (hydroxyapatite) (Kim 2015; Choi and Ben-Nissan 2019).

Up to recent years, these materials have been mainly employed for the production of low economic value products such as pet food, fish silage, fertilizer, and fish oil (Mondal et al. 2019; Nagai and Suzuki 2000; Senaratne et al. 2006). Recently, however, they have been enjoying interest from researchers for developing value-added advanced materials each with significant potential for use in bone, skin, cardiovascular, liver, and other tissue engineering and drug delivery applications (Clarke et al. 2011; Komalakrishna et al. 2017; Jovic et al. 2019).

Hence, the global marine biotechnology market is expected to grow from USD 3.5 billion in 2017 to USD 6.5 billion in 2024, at a compound annual growth rate of 9.61% mainly due to the rising production and usage of these high-end products in various industries and growing expenditure in R&D activities (Energias Market Research 2019). In the following sections, a concise review of selected biomaterials of marine origin will be presented *with special emphasis on synthesis, characterization, and application*.

7.2.1 Polysaccharides

Polysaccharides are polymeric carbohydrate structures, usually composed of various monosaccharides linked with different glucosidic bonds. They widely exist in

animals, plants, microorganisms, and algae (Yang and Zhang 2009; Jeon et al. 2011). Chitin or chitosan is an example of animal-based polysaccharides, while agarose, alginate, carrageenan, and fucoidan are nonanimal groups of polysaccharides extracted from algae. These polysaccharides that have been subject to significant interest in tissue engineering applications in recent years are described in more detail in the following paragraphs.

Agar/agarose: Agar that is a gelatin-like polysaccharide made primarily from the red algae is used as a solidifying component of bacteriological culture media and canning meat, fish, and poultry. It is also used in cosmetics, medicines, and dentistry. Agarose, the main component of agar, is extracted by the extraction of agaropectin from agar (Scionti et al. 2014; Shin et al. 2010). It is another natural carbohydrate polymer with excellent characteristics though it has received relatively less attention in biomedical applications compared to other polysaccharides such as alginate and chitosan (Rahmati et al. 2016; Xia et al. 2017; Zarrintaj et al. 2018). Agarose is released on boiling at temperatures around 80 to 90 °C, forming a thermally reversible gel upon cooling to around 30 to 40°C as a result of extensive hydrogen bonding between the agarose chains. Both the release and gelling temperatures vary depending on the molecular weight and monomer compositions (methoxyl content) (Gustavsson and Larsson 2003).

Owing to the time-dependent mechanical properties of the formed gel, the stress relaxation behavior of agarose gel is similar to native tissue (Buschmann et al. 1992). Therefore, agarose has been used extensively for the encapsulation of cells for cartilage tissue engineering (Xiong et al. 2005; Roach et al. 2016). Other tissue engineering applications of agarose are in bone regeneration (Lopez-Heredia et al. 2017), wound healing (Awadhiya et al. 2017a), artificial pancreas (Iwata et al. 1992), and cardiac regeneration (Mayfield et al. 2014). Also, since the gel-forming proceeds through hydrogen bonding, agarose is a good biocompatible polymer candidate since there is no need for use of toxic cross-linking agents like genipin (Sarem et al. 2013; Cecilia et al. 2017; Campos et al. 2018). Zarrintaj et al. (2018) provide an extensive review of the applications of agarose-based biomaterials in tissue engineering.

Agarose has few drawbacks in tissue engineering applications; however, one of which is the less-than-optimal degradation behavior since it forms very stable hydrogels in vitro and in vivo. Another is the lack of receptors in mammalian cells to bind to the agarose, which leads to a lack of interaction between agarose and encapsulated cells (Roberts and Martens 2016). Although agarose exhibits the poor cell attachment, it has been reported by Zarrintaj et al. (2018) that blending or coupling agarose with other polymers and proteins such as collagen, chitosan, and bacterial cellulose increases cell affinity to agarose (Miguel et al. 2014; Annamalai et al. 2016; Awadhiya et al. 2017b).

Alginate: One of the most abundant marine biopolymers, alginate, is extracted from the cell walls and intracellular spaces of brown seaweeds. Alginate is an unbranched block copolymer containing mannuronic acid and guluronic acid residues in different proportions. Critical factors affecting the physical properties of alginate are the ratio and arrangement of mannuronic and guluronic acid residues

and molecular weight. For example, alginates with higher guluronic block ratios form rigid gels with increased Ca2 + ion. However, calcium alginate gels with higher mannuronic blocks are soft and elastic.

Alginate can form a physical gel by hydrogen bonding at low pH or by ionic interactions with polyvalent ions like Ca, Sr, and Al. Guluronic blocks, which are mainly responsible for such ionic interactions, selectively couple with Ca2+ to form a row of reversible and strong electrostatic cross-links and so form egg-box type aggregates (Grant et al. 1973; Li et al. 2007; Cao et al. 2020; Goh et al. 2012).

Alginate is used in many industrial applications owing to its attractive chemical and physical properties. For example, it is used as a thickener and stabilizer agent in the food and beverage industry (drinks, jelly, ice-cream), fabrics, paper, paint, and toothpaste industries; as an encapsulation material in cell culture and transplantation application and ethanol production; and as a mold in a dental application. It is also widely used in tissue engineering and drug delivery application due to its chemical and physical modifiability. It could be produced as microspheres, microcapsules, hydrogels, sponges, foams, and fibers and can be combined with active molecules such as growth factor or peptides for the specific tissue applications (Goh et al. 2012; Venkatesan et al. 2015; Fernando et al. 2019; Chen et al. 2020b).

Alginate has been combined with many other natural and synthetic polymers, ceramics, and bioglass to produce composite scaffolds in bone tissue engineering application (Marsich et al. 2013; Sajesh et al. 2013; Sowjanya et al. 2013; Venkatesan et al. 2014a). Among these, chitosan is most commonly used to produce composite alginate scaffolds for bone regeneration. Venkatesan et al. (2014b) have presented an excellent review of the classification of composite alginate scaffolds based on the type of natural polymers.

Alginate is also considered a promising material for wound healing and skin tissue regeneration due to its advantageous properties, such as protecting from bacterial infection, low toxicity, gelling properties maintaining a moist microenvironment, facilitating wound healing, and combinability with biologically active molecules (Pereira et al. 2013; Aduba and Yang 2017; Zhou et al. 2018; Feng et al. 2020). It can form hydrogels under very mild conditions, at room temperature, and in the absence of toxic solvents, especially calcium alginate hydrogels stand out in wound healing. Ionic cross-linking, covalent cross-linking, thermal gelation, and cell cross-linking are typical methods to form hydrogels. The properties of hydrogels largely depend on the molecular weight and chemical composition of the polymers, cross-linking type, and density (Lee and Mooney 2012). In addition, alginate is among the most promising matrices for use as bio-ink for 3D printers.

Carrageenan: Carrageenans are another family of linear sulfated polysaccharides extracted from red edible Chondrus crispus species of seaweed, also known as Carrageen Moss or Irish Moss. They have been used as a home remedy for coughs and cold and in food preparation since 400 AD. They are ingredients in many organic and vegan foods and are widely used in the food industry for their gelling, thickening, and stabilizing properties and have been patented in the USA as a food additive since the 1930s. Since they strongly bind to food proteins, carrageenans are also widely employed by the dairy and meat industries. Carrageenans are also used

in experimental medicine, pharmaceutical formulations, cosmetics, and industrial applications (Necas and Bartosikova 2013). They have been used as a laxative, as a treatment for peptic ulcer disease, and as a component of pharmaceuticals, toothpaste, aerosol sprays, and other products (Tobacman 2001).

Carrageenan is produced by one of the two main methods: The first method had been used late 1980s where the carrageenan was extracted from the seaweed into an aqueous solution, the seaweed residue was removed by filtration, and then the carrageenan is recovered from the solution, eventually, as a dry solid containing little else than carrageenan. In the second method, the seaweed is treated in an alkaline solution in order to dissolve everything else other than carrageenan and other insoluble matrices, which is mostly cellulose. The insoluble residue that consists largely of carrageenan is then dried and sold as semi-refined carrageenan. This method is more practical and economical since it does not involve dissolution and recovery steps for the carrageenan.

The three main varieties of carrageenan are kappa, iota, and lambda carrageenans. These compounds differ in their degree of sulfation extent of branching, solubility, cation binding, and ability to form gels under different conditions though they all have similar D-galactose backbones (alternating α1,3 to β-1,4 linkages). Kappa-carrageenan has one sulfate group per disaccharide, iota-carrageenan has two, and lambda-carrageenan has three (Klose and Glicksman 1968).

There has been an increasing interest in the use of carrageenan in drug delivery and tissue engineering applications (Yegappan et al. 2018). Carrageenan has been employed in recent studies as a scaffold to produce a macroporous cryogel matrix with good mechanical strength and viscoelastic behavior (Sharma et al. 2013), as a scaffold for in vitro culture of human skin-derived multipotent stromal cells (Rode et al. 2018), for soft tissue repair in breast cancer (Tytgat et al. 2019), as bone repair material (Daniel-Da-Silva et al. 2007; Kim et al. 2011; Feng et al. 2017), and in cartilage tissue engineering (Bhattacharyya et al. 2010; Popa et al. 2015).

It should be mentioned that there has been a controversy on the use of carrageenans as a food additive since there have been indications that carrageenan is highly inflammatory and toxic to the digestive tract and that it may be responsible for colitis, IBS, rheumatoid arthritis, and even colon cancer (Tobacman 2001). The International Agency for Research on Cancer (ARC, 1883) designated degraded carrageenan as group 2B based on animal models, which prompted several researchers advising the use of carrageenan in food (Watt and Marcus 1981; Ekström et al. 1983; Ekström 1985).

Chitosan: This is a biopolymer, which is a deacetylated derivative of chitin, which is the main source of chitosan, and is available in the shells of crustaceans, insects, and fungi (Ali and Ahmed 2018; Ahmed and Ikram 2015). Chitosan is flexible for chemical modifications due to the presence of reactive amino and hydroxyl group in the molecular chain; therefore, its structural versatility allows it to be applicable for tissue engineering-centered applications (Rodríguez-Vázquez et al. 2015; Ahmed et al. 2018). Being easily processable, chitosan can be chemically or physically modified to manufacture tailor-made functional forms such as gels, nanofibers, membranes, beads, nanofibrils, nanoparticles, microparticles, scaffolds,

and sponge-like structure with desired properties (Subramanian et al. 2005; Sanyakamdhorn et al. 2013; Tang et al. 2014). Also, both chitin and chitosan show high bioactivity, biodegradability, biocompatibility, nontoxicity, and antimicrobial characters similar to the other biopolymers.

Chitosan-based materials are used in many different applications in tissue engineering. They are the preferred materials in cartilage tissue engineering because of chitosan's similarity with glycosaminoglycans found in ECM, which can stimulate cartilage chondrogenesis. Chitosan can interact electrostatically with negatively charged glycosaminoglycans and contribute to their synthesis. Chitosan has also be added to other materials such as collagen, hydroxyapatite (Kaviani et al. 2019), gelatin (Shen et al. 2015; Kuo et al. 2015), and silk fibroin (Li et al. 2019) to produce composite structures, which are preferred for cartilage tissue engineering.

Though it is one of the most commonly used natural polymers in bone tissue engineering applications, chitosan scaffolds, which have lower mechanical strengths than the normal bone, cannot satisfy the load-bearing requirements for such structures. Also, similar to copper or silver-doped bone cement implants (Albrektsson and Johansson 2001), chitosan scaffolds do not have favorable osteoconductive properties (Zhang et al. 2003; Rodríguez-Vázquez et al. 2015; Islam et al. 2020). Hence, there have been attempts to increase the mechanical strength and structural integrity of chitosan scaffolds by creating composite structured with other materials. Many researchers created chitosan composites with hydroxyapatite to mimic natural bone by improving its mechanical properties and osteoconductivity (Li et al. 2002; Kong et al. 2005; Frohbergh et al. 2012; Venkatesan et al. 2012; Lima et al. 2013; Lei et al. 2017; Balagangadharan et al. 2018). Kar et al. (2016) have included Montmorillonite to the hydroxyapatite / chitosan composite scaffold to eliminate the brittleness caused by hydroxyapatite and improve the mechanical strength. Shavandi et al. (2015) produced chitosan/hydroxyapatite/β-tricalcium phosphate composite scaffolds with chitosan from squid and crab and hydroxyapatite and β-tricalcium phosphate from waste green mussel shells. Some other materials employed to form composite structures with chitosan are collagen, polyethylene glycol, polycaprolactone, poly(propylene carbonate) polylactic acid, alginate, fucoidan, and silk fibroin (Bhardwaj and Kundu 2011; Jing et al. 2015; Shakir et al. 2015; Puvaneswary et al. 2015; Kozlowska et al. 2019). There are also more recent studies that produced pure chitosan foams with much better mechanical behavior (Polat et al. 2020a, b).

Besides bone tissue engineering, chitosan with its excellent antimicrobial properties is also used in forming protective coatings on dental implants and in drug delivery in bioidentical applications (Husain et al. 2017). In addition, 3D chitinous scaffolds have been produced via decellularization and demineralization of the demosponge exoskeleton as an attractive approach for designing tissue engineering scaffolds via biomimetic strategies (Mutsenko et al. 2017a; Mutsenko et al. 2017b; Binnewerg et al. 2020). Demosponge that belongs to the Aplysinidae family is of interest as the producer of bromotyrosine (bioactive secondary metabolites) and chitinous scaffolds (Kunze et al. 2013; Wysokowski et al. 2013). Demosponges, which have highly porous and complex canal systems that allow the

diffusion of nutrients, oxygen, and the removal of waste products and pose as a potentially renewable resource due to the ability to grow under marine farming conditions, provide a suitable structure necessary for the adhesion and proliferation of cells (Rohde and Schupp 2011). The recent reviews by Saravanan et al. (2016), Ahmed et al. (2018), and Islam et al. (2020) describe extensively the many different applications of chitosan in biomedical applications.

Fucoidan: Another marine source material, which plays a vital role in biomedical applications is fucoidan (Jeon et al. 2011), which is a class of sulfated polysaccharides, mainly composed of fucose. They can be found in various species of brown algae and brown seaweed (Cunha and Grenha 2016) and are already used in the food and dietary supplements and cosmetic industry. Their application in various biomedical areas is increasing because of their water solubility, biological activities, and negatively charged sulfate groups, which impart them with features such as inhibition of acute and chronic inflammation, anticancer, antitumor, anticoagulant, and antioxidant activities, supporting angiogenesis, and promoting the proliferation of fibroblasts (Kannan et al. 2018; Oliveira et al. 2020).

Fucoidan can also be a promising candidate for artificial bone development. Cho et al. (2009) have shown that the fucoidan extracted from the marine brown algae induces osteoblastic cell differentiation and affects bone formation, remodeling, and mineralization positively. Many other studies have also shown that fucoidan and composite fucoidan materials facilitate the proliferation and migration of fibroblast cells, promote angiogenesis, and affect therapeutic revascularization positively (Luyt et al. 2003; Changotade et al. 2008; Park et al. 2012; Kim et al. 2018; Chaves Filho et al. 2018; Perumal et al. 2018). For bone tissue engineering, fucoidan has been combined with different materials such as poly(3-caprolactone) (Jin and Kim 2011) and collagen (Perumal et al. 2018). The particularly prominent composite material, however, is the combination of fucoidan with chitosan and/or hydroxyapatite (Nakamura et al. 2008; Jeong et al. 2013; Puvaneswary et al. 2015; Lowe et al. 2016; Lu et al. 2018; Lu et al. 2019; Chen et al. 2019).

7.2.2 Proteins (Collagen)

Collagen, the most abundant structural protein of connective tissues in mammals, already finds use in cosmeceuticals, nutraceuticals, pharmaceuticals, and functional food. It is also widely used in the biomedical area, especially in bone-related diseases (Asghari et al. 2017; Fernando et al. 2019) in the forms from injectable collagen solutions to bone regeneration scaffolds (Pallela et al. 2013). Collagen interacts with a large number of molecules and ECM components and contributes to regulating many cellular processes including adhesion, proliferation, migration, and also plays a critical role in a matrix structure and remodeling (An et al. 2016; Leitinger and Hohenester 2007).

Industrial collagen production mostly relies on extraction from animal tissues of cows, pigs, and sheep due to high sequence homology with human collagen. However, the use of collagen derived from mammals and recombinant production

systems is limited since it poses a risk to trigger an immune reaction (in about 3% of the population) and spread the infectious diseases dangerous to humans (Avila Rodríguez et al. 2018). Specifically, collagen from a bovine source carries a high risk of encephalopathy or transmissible spongiform encephalopathy. In addition, the utilization of porcine and bovine collagen leads to cultural or religious concerns in some populations (Salvatore et al. 2020). The production of collagen with recombinant technology is also limited since the full-length collagen cannot be obtained with native amounts of post-translational modifications for prokaryotic and eukaryotic hosts (Wang et al. 2017a).

Collagen extracted from discards or by-products of fish processing (such as skin, bones, heads, guts, and scales) (Nagai and Suzuki 2000), jellyfish (Nagai and Suzuki 2002; Kittiphattanabawon et al. 2005; Song et al. 2006), sea urchin (Benedetto et al. 2014), starfish (Lee et al. 2009), muscles and skins of marine animals (Sikorski and Borderias 1994), squid skins (Kołodziejska 1999), and sponges (Swatschek et al. 2002; Tziveleka et al. 2017; Garrone et al. 1975; Pallela et al. 2011; Swatschek et al. 2002) display the same properties as the mammal-extracted collagen but have a much superior bioavailability (Kim and Mendis 2006). These sources provide abundant, cost-effective, and eco-friendly sources of collagen extraction (Liu et al. 2015b; Senaratne et al. 2006).

Fish collagen that finds use in pharmaceutics, cosmetics, food industry, and edible coatings because of its biocompatible, easily accessible, and very low or no possibility of zoonosis (Jeevithan et al. 2013; Yamada et al. 2014) has been extensively studied for use in tissue engineering applications in the literature (Yamamoto et al. 2015; Sun et al. 2017; Salvatore et al. 2020). Some specific examples of its use can be found in bone (Sugiura et al. 2009; Mansouri et al. 2019) and skin (Fernández-Cervantes et al. 2020) replacement and wound dressing (Cao et al. 2015; Zhou et al. 2015; Jridi et al. 2015; Zhou et al. 2016). Also, hybrid/composite scaffolds were produced to overcome the limiting weak mechanical properties of collagen and improve the biological properties of the scaffolds (Chandika et al. 2015; Raftery et al. 2016; Coelho et al. 2017; Ullah et al. 2018; Govindharaj et al. 2019). The skeletons of marine sponges, especially demosponges, are also used for the production of bone tissue grafts due to their highly porous structure and supporting growth of human osteoprogenitor cells (Green et al. 2003; Zheng et al. 2007; Nandi et al. 2015; Clarke et al. 2016; Lin et al. 2011).

7.2.3 Bioceramics (Hydroxyapatite)

Hydroxyapatite (HAp) is a naturally occurring mineral, belongs to the apatite family, and can be derived from plants (reefs, algae, leaves, stalks), animals (bovine bone), biogenic (eggshells, snail, and seashells), and marine sources (corals, fish, crustaceans). In terms of HAp production from plants, the leaves, stalks, and flowers of mint, green tea, khat, drumstick tree, clover, and basil and from the orange and potato peels and effluents from fruit processing plants are primary sources (Nayar and Guha 2009; Tampieri et al. 2009; Shaltout et al. 2011; Akram et al. 2014;

Govindaraj and Rajan 2016). Marine algae are also used for the synthesis of hydroxyapatite through pyrolysis and hydrothermal synthesis due to their calcite ($CaCO_3$) content, which is similar to human bones with their interconnectivity and porosity (Walsh et al. 2008; Teymouri et al. 2018).

Marine sources include fishbone (Mondal et al. 2019), sponge and corals (Kusmanto et al. 2008), shells (Pal et al. 2017), and cuttlefish (Milovac et al. 2014) and also offer a lot of variety for HAp extraction. Different methods are used to produce HAp depending on the source, calcination, polymer-assisted methods, subsequent alkaline hydrolysis, and thermal treatment being the common routes (Lalzawmliana et al. 2019).

Biogenic sources can include inexpensive and easily accessible egg shells, snail shells, and seashells. Marine-derived seashells, for example, mollusk shells, have high mechanical strength compared to other biogenic sources (Agbeboh et al. 2020; Mondal et al. 2019).

Though HAp can be synthesized chemically through precipitation, hydrolysis, sol–gel, emulsification, mechanochemical, and hydrothermal synthesis (Agbeboh et al. 2020), HAp produced from natural sources has better biochemical properties (Shi et al. 2018). HAp of biological origin is enriched in elements such as Mg^{2+}, K^{+}, and Na^{+}, which play a role in bone repair, and has a relatively lower crystallinity than synthetic ones (Leventouri 2006). Large quantities of calcium- and hydroxyapatite-rich discards resulting from increasing fish and crustacean consumption also make HAp from marine sources more environmentally friendly and economical. The HAp synthesis from crude blue shark jawbone is an example of an environmentally friendly production method (López-Álvarez et al. 2020).

The human bone is composed of mineral (60 to 70% hydroxyapatite) and an organic (primarily collagen) phase where the collagen serves as a matrix in the organic phase for the precipitation of HAp (Constantz et al. 1995; Felício-Fernandes and Laranjeira 2000). Hence, HAp is considered to be the most suitable bone replacement element in bone tissue engineering applications (Sun et al. 2011; Shi et al. 2018). At physiological pH and temperature, HAp facilitates the formation of new bones by deposition of calcium and phosphate ions on new bone tissues and promotes the biological activities of osteoblasts.

In bone tissue engineering, the material should be designed in accordance with the type and mechanical properties of the bone and should have similar mechanical and biological properties. Although HAp has excellent osteoconductivity, its brittleness and relatively slow degradation rate are restrictive features for its use in this area. To overcome these disadvantages, the production of composite materials using synthetic or natural polymers has come forward where HAp helps to improve mineralization, and increases cellular activity and differentiation in these composites (Juhasz et al. 2010; Kim et al. 2015). Considering that bone is composed of 70% minerals (HAp crystals) and 30% organic matter like collagen, glycoproteins, and proteoglycans, collagen and Hap composites should have a very close compositional similarity to natural bone (Salgado et al. 2004). The studies by Villa et al. (2015), Chen et al. (2016), and Tien et al. (2012) can be given as examples of HAp/collagen composite scaffolds. Another example is the HAp-polylactic acid composite

scaffolds to facilitate cell attachment and proliferation on the surface of the material (Mondal et al. 2020). Milovac et al. (2014) showed that the coating of cuttlefish-derived HAp with poly(ε-caprolactone) improved the mechanical properties of the scaffold. In addition to this study, PCL/HAp composite materials obtained through different synthesis routes were also investigated in some other studies (Kim et al. 2004; Kusmanto et al. 2008; Zhao et al. 2010). Chitosan is also widely used in a blend with HAp to create composite scaffolds in bone tissue engineering. These studies are discussed above under the title of chitosan.

7.3 Typical Scaffold Fabrication Methods

A proper scaffold material in any tissue engineering application should support cell proliferation, vascularization, migration, and tissue formation, and should degrade naturally during or after the healing process. Considering that the scaffold material should also be biocompatible and nontoxic, display desired durability when necessary, and evade the immune response of the host, it is expected to possess certain biological, structural, and chemical properties as mentioned previously (see Fig. 7.2). It is a priori that the path to achieving these properties starts with choosing the appropriate material for the system in question. However, it should be stressed that the synthesis method employed to create the scaffold material is as important as the selection of the material to attaine the features required.

The various methods and techniques developed to create scaffolds pose different advantages or disadvantages. It is important to realize that many of these methods are actually variations of one of the main routes to creating scaffolds. A good example is a template–polymerization route that employs nano- or micron-sized template particles dispersed in an organic solvent–polymer solution. The template materials can be gas, liquid, or solid. Polymerization can be achieved through solvent removal by freeze-drying or evaporation, or by use of a cross-linking agent. The leftover polymer–porogen matrix is then treated with weak acid, hot water, or by other means to reach out (in case of a solid porogen) or evaporate (in case of a liquid porogen) the porogen to obtain a porous scaffold structure. If the porogen is gas, no porogen removal may be necessary.

The methods for scaffold synthesis for tissue engineering described below have been grouped as conventional and advanced fabrication techniques, and their discussion has been carried out without a change in the terminology in order to make comparison with the literature easier. Two excellent recent reviews on these techniques are provided by Eltom et al. (2019) and Mabrouk et al. (2020). A summary of these techniques and advantages/disadvantages is also presented in Table 7.2.

Table 7.2 A summary of the techniques used for manufacturing tissue engineering scaffolds

Fabrication technique	Advantages	Disadvantages
Solvent casting/particulate leaching	• Controlled pore size/ porosity • Wide material diversity	• Limited membrane thickness • Residual solventLack of mechanical strength
Fiber bonding	• Highly porous/ interconnected pores • Large surface area • Easy processing	• Limited applicability • Residual solvent • Lack of mechanical strength
Rapid prototyping	• Pore size gradient • Rapid production	• Expensive equipment • Lack of material diversity • Limited mechanical strength
Melt molding	• Limited control of pore size/porosity	• High temperature
Freeze-drying (lyophilization)	• Manageable pore size • Wide applicability	• Irregular pore size • Residual solvent • Small production amounts
Gas foaming	• Solvent-free	• Disordered porosity • Limited mechanical strength
Electrospinning	• Simple/cheap processing • Large surface area	• Limited mechanical strength • Residual solvent

7.3.1 Conventional Techniques

Solvent casting and particulate leaching (solid templating): The solvent casting/particle leaching technique depends on initially dissolving the polymer in an organic solvent. Then, water-soluble solid porogen particles of some salt such as sodium chloride, tartrate, or citrate of certain diameters are added into this solution (Yang et al. 2006; Bartis and Pongrácz 2011; Sampath et al. 2016; Mabrouk et al. 2020). The polymer mixture is then poured into a mold, and the solvent is removed through freeze-drying or evaporation allowing the material to form a composite matrix of porogen particles and polymer. The porogen particles are then dissolved away from the matrix by immersion in weak acid or water, leaving behind a porous polymeric structure. The pore sizes range from 5 to 600 μm with irregular microstructures, pore size, and its interconnectivity depending on the size of the porogen particles. Though it is inexpensive, easily applicable, and does not require additional special equipment, the method has the disadvantages that removing the porogen from the core of the material can be difficult and the solvent residues, which may remain in the structure, may be detrimental to the cells. Different porogen, polymers, composites, and combinations with other techniques have been tested to overcome the stated problems (Fonseca-Santos and Chorilli 2017; Jahed et al. 2017; Agrawal et al. 2017; Preethi et al. 2017; Thadavirul et al. 2013). For bone engineering applications, different concentrations of ammonium bicarbonate were tested as porogen in polylactic acid/hydroxyapatite/surfactant (polyethylene oxide) mixture to obtain a porous nanocomposite. However, in the presence of ammonium bicarbonate, the

increase in the porosity of the nanocomposite was associated with decreases in the mechanical properties (Thanh et al. 2016).

Melt molding (solid templating): In this technique, gelatin microspheres are mixed with a specific polymer powder (such as polylactic glycolic acid due to its lower glass transition temperature) in a mold and the mixture is heated above the polymer glass transition temperature. The resulting composite is placed in water after heating to dissolve the water-soluble microspheres resulting in a porous structure. Although the pore size and porosity can be easily controlled with the porogen concentration and diameter, respectively, the lack the uniform arrangement of gelatin microspheres through the polymer solution is the disadvantage of this method. On the other hand, this technique does not require the use of toxic solvents and it is suitable for combining with other methods. (Mao et al. 2018).

Emulsification polymerization/freeze-drying (liquid templating): This method is based on the homogenization of a polymer-organic solvent solution in water to create an emulsion. The resulting mix is then rapidly cooled and the water/solvent is removed by freeze-drying. The scaffold formed this way has high porosity, interconnectivity, various sizes, and shapes. The pore size of the scaffold ranges from 20 to 200 μm and may have more than 90% porosity. In this method, water and solvent act as the porogen. The pore sizes are controlled by changing such parameters as the freezing rate, solution pH, polymer amount in solution, and temperature.

In another variation of this technique, an oil-in-water emulsion of hexane is created in the acetic acid–chitosan solution. Chitosan is polymerized by the addition of a cross-linking agent, locking the hexane droplets in the polymer matrix. The system is then frozen rapidly in a lyophilizer and the oil is removed by freeze-drying, leaving a porous structure with good interconnectivity (Polat and Polat 2020; Polat et al. 2020a; Cihan et al. 2017)

The advantage of this technique is that it does not require high temperatures or separate leaching steps. This prevents the effects of integrated biological factors that come from high temperatures. Eltom et al. (2019) state that the techniques may have some disadvantages such as having some solvent residue in the foam structure, irregularly sized pores, and restriction to small quantities of production. It should be noted that Polat et al. (2020a) could determine no hexane in the structure of the residual chitosan foam in FTIR analyses following the freeze-drying step.

Gas foaming (gas templating): In this technique, a high-pressure gas (such as N_2 or CO_2) is applied to a chamber filled with scaffolding material for a certain time to saturate the polymers with gas. Intermolecular interactions between CO_2 and polymer molecules increase, and rapid depressurization causes thermodynamic imbalance. This causes the gas molecules to clump together and act as templates for the pores, which form in the polymer matrix. The pressure of gas determines the porosity, swelling ratio, compression properties, and elastic modulus (Annabi et al. 2010). A scaffold with approximately 100 μm pore diameter and up to 93% porosity can be formed.

The main advantage of this method is the elimination of the use of a solvent. However, the method yields heterogeneous pore structure, and controlling the pore

connectivity and pore size is also very difficult (Poursamar et al. 2015; Sampath et al. 2016). To eliminate the associated problems and create pores with different sizes, salt particles at different concentrations and sizes have been also used as porogen in the polymer solutions (Bak et al. 2014).

Natural or synthetic polymers and their composites with inorganic biomaterials such as hydroxyapatite and bioactive glass have been widely used in the freeze-drying techniques (Tohamy et al. 2018; Boulila et al. 2017). In some studies, this technique was combined with other methods to increase the biomedical potential of biomaterials such as gas foaming (Kazimierczak et al. 2020), high-speed stirring (Maji et al. 2018), and electrospinning (Yuanyuan and Song 2012; Kim et al. 2013).

Thermally induced phase separation: The method is based on liquid–liquid phase separation and is the most promising, flexible, and tunable route for the preparation of 3D interconnected polymeric porous structures (Liverani et al. 2019). In this method, a polymer is dissolved at elevated temperatures in a low molecular weight solvent with a high boiling point to form a homogeneous solution. When the hot polymer solution is cast onto a mold and cooled, the system goes through phase separation and is solidified into distinct polymer-rich and polymer-poor phases. Then extraction, dissipation, and sublimation steps are followed in order to eliminate the solvent used, which produces a 3D microporous polymer matrix (Mabrouk et al. 2020). If solid bioactive particles are also included in the system, the porous structure is also impregnated with these particles.

The structure may be adjusted by controlling several factors including the polymer characteristics and solvent. It is claimed that the advantage of this method is to produce structures with good mechanical properties and controlled pore morphology (Garg et al. 2012; Sampath et al. 2016; Mabrouk et al. 2020). The kinetics of the phase separation controls the morphology and pore distribution of the foam. The resulting pore diameters range from 50 to 100 μm. The polymer concentration affects the porosity percentage that can reach up to 87%. The usage of organic solvents that might have a toxic effect on cells, on the other hand, is the limitation of this technique.

7.3.2 Advanced Fabrication Techniques

Electrospinning: Electrospinning is a versatile technique, which can employ a variety of natural polymers and composites and abundant synthetic polymers. Scaffolds with high porosity and surface area can be manufactured by electrospinning. It is suitable for different material combinations and thickness, porosity, and aspect ratio of the scaffold that can be easily controlled. An electrospinning assembly consists of a high voltage control source, a nozzle, and a metallic collector (for electrospun fibers). The polymers are mixed in a suitable solvent homogeneously, and this solution is brought into the metal nozzle and a high electric field is applied. When the voltage reaches a critical value, the jet droplet of the solution in the tip of the syringe is electrically charged and collected as continuous fibers on the grounded collector plate. The properties of nanofibers depend on

several critical parameters such as solution parameters (viscosity/concentration, conductivity/solution charge density, surface tension, and polymer molecular weight), process parameters (feed rate, voltage, a distance of tip and collector, needle tip design and placement, collector composition, and geometry), and ambient conditions (temperature, humidity). For example, solution concentration and feed rate are factors that determine the diameter and morphology of the fibers. Smaller diameter fibers can be obtained as the solution concentration and feed rate are reduced or the applied voltage, solution conductivity, and distance between the tip and the plate are increased (Pham et al. 2006; Mabrouk et al. 2020; Sill and Von Recum 2008). Many different types of collector systems such as parallel plates (Fridrikh et al. 2003), cylindrical drums (Chew et al. 2005), and modified rotating disk (Yee et al. 2008) have been used in the literature.

Rapid prototyping: Rapid prototyping (RP) (freeform fabrication or additive manufacturing) is the common name for several advanced techniques that produce objects from computer-aided design data in a layer-by-layer manner. This technology is widely used industrially and in tissue engineering to produce fast and consistent scaffolds. Scaffold production stages can be categorized under 3 main steps with RP techniques: three-dimensional scaffold design, two-dimensional slicing, and RP fabrication.

The main advantage of the RP technique is its ability for rapid scaffolding production with consistent quality, texture, and structure. However, the technology requires special and expensive equipment, high processing temperatures, and multidisciplinary collaboration. Common RP techniques for tissue engineering scaffolds fabrication are extrusion-based RP techniques, three-dimensional printing, selective laser sintering, three-dimensional bioprinting, stereolithography, melt deposition, electron beam melting, and selective laser melting. Among these, stereolithography is one of the oldest techniques and is based on obtaining a solidified 3D model of a photocurable liquid polymer using a laser beam guided by a computer. Many research groups have used this technique to produce scaffolds with desired mechanical properties and porosity (Arcaute et al. 2010; Melchels et al. 2009; Melchels et al. 2010; Martínez-Vázquez et al. 2015). There are excellent review papers by Abdelaal and Darwish (2013), and Yeong et al. (2004) classify rapid prototyping techniques and summarize the details and compare their advantages and disadvantages.

3D Printing: The basic processing principle of 3D bioprinting for biomedical and tissue engineering applications is the same as traditional 3D printers employing an inkjet printing approach with the main difference that a combination of cells, biomaterials, and/or growth factors is employed as ink. A digitally created model is transformed into a 3D object by layer-by-layer rasterizing. The major bioprinting methods are extrusion-based, inkjet, stereolithography-based, and laser-assisted bioprinting methods (Derakhshanfar et al. 2018). Alginate and collagen have been employed as bio-inks in cartilage (Yang et al. 2018), bone (Kim et al. 2016; Ojansivu et al. 2019) and nerve (Gu et al. 2016; Ning et al. 2018), and general tissue engineering (Irvine et al. 2015; Kesti et al. 2015; Wu et al. 2016; Lee et al. 2016; Colosi et al. 2016) since it mimics natural ECM, low cytotoxicity, high water content, biodegradability, printability, and cross-linkability. The study of Jakus

and Shah (2017), Senatov et al. (2016), and Trachtenberg et al. (2017) can be given as examples of HAp composites produced by 3D printing for tissue engineering applications. The state of art in 3D printing can be found in the reviews by Li et al. (2014) and Wang et al. (2020).

7.4 Marine-Based Scaffold Systems

Scaffolds are engineered forms or structures, which provide an environment for desired cellular interactions to proceed toward the formation of new three-dimensional functional tissues in regenerative medicine. Owing to the diversity, availability, and sustainability of the sources it offers, marine sources are attractive candidates for scaffolding applications in biotechnology, specifically in tissue engineering. Numerous organisms present in the marine environment also exhibit tested and tried forms and functionalities, which inspire the development of novel ideas and products. Hence, an increasing number of compounds have been isolated from marine organisms, especially in recent years for the purpose of developing products, which can be employed to substitute the synthetic ones traditionally employed in regenerative medicine. The process of development requires the production of highly proficient structures, which will be able to function at the nano-, micro-, and macroscopic levels for eventual clinical success. These structures should be able to be transformed into scaffold systems, which satisfy the requirements summarized in Fig. 7.2, and should interact properly with the components presented in Fig. 7.1. Since there is an immense amount of diversity of both raw materials and structures, the most reasonable strategy is to copy from the existing designs in the marine environment and modify it as necessary to obtain the desired functionality (Ben-Nissan and Green 2013; Ben-Nissan 2015).

There are numerous studies that describe scaffolds in tissue engineering, which forces classifications of these structures which can be done based on the starting raw materials, the fabrication method employed, or depending on the form of the resulting scaffold structure (Dhandayuthapani et al. 2011).

Since the types of the starting materials are described in Sect 7.2 and the types of synthesis techniques are given in Sect. 7.3, the most common forms (hydrogels and porous matrix or foams) will be presented in the following paragraphs.

Hydrogel Scaffolds: Hydrogels are a three-dimensional network of hydrophilic polymers used in a wide variety of applications in the biomedical fields including drug delivery, wound healing, cosmetics, and tissue engineering. The most prominent property of a hydrogel is its ability to swell and hold a large amount of water (>10%) while maintaining its structure through chemical or physical cross-linking of the hydrophilic amino, carboxyl, and hydroxyl groups in its polymer chains (Singh et al. 2016).

Hydrophilic polymers are termed either physically or chemically cross-linked hydrogels depending on whether the cross-linking is through intermolecular attractions or covalent bonding. Physically cross-linked, or self-assembly, hydrogels are formed when they self-assemble through noncovalent secondary molecular

interactions and through extended linkage regions of several laterally related polymer chains. Different methods such as ionic interactions, crystallization, heating or cooling a polymer solution, hydrogen bonding, complex coacervation, and protein interactions are employed to manufacture physically cross-linked gels (Singhal and Gupta 2016; Ranganathan et al. 2018; Xiang et al. 2020). The absence of cross-linking agents and the relative ease of manufacture are the advantages of physical cross-linking (Hunt et al. 2014; Maitra and Shukla 2014). On the other hand, the network formation is random and physical interactions that can be reversed under different physical conditions or stress, thus causing structural deterioration of the hydrogels.

Chemical hydrogels contain covalent bonds between different polymer chains, in this way hydrogels with a wide range in network structures and good mechanical stability can be produced. Methods like radical polymerization, chemical reaction of complementary groups, using enzymes, and high energy irradiation are employed to synthesize chemically cross-linked hydrogels (Hennink and van Nostrum 2012). However, toxic cross-linking agents that are harmful to human health must be completely removed from hydrogels in biomedical applications (Ranganathan et al. 2018; Hennink and van Nostrum 2012).

It should be noted that hydrogels may be assigned different names in the literature depending on their source, polymeric composition, physical appearance, and network electrical charge (Ahmed 2015; Elsayed 2019; Singhal and Gupta 2016). For example, a classification based on their physical appearance, which results from the polymerization technique used in their production, may lead to structures named "matrices, films, or microspheres."

Hydrogels provide unique advantages compared to other polymeric scaffold types since they are very biocompatible, possess flexibility very similar to natural tissue, and have excellent permeability for the transfer of oxygen, nutrients, and other water-soluble metabolites due to their high water content (Hunt et al. 2014; Caló and Khutoryanskiy 2015; Pan et al. 2015; Koehler et al. 2018; Mantha et al. 2019; Aswathy et al. 2020). They act as a space-filling agent and function as a biological glue, providing bulking, preventing adhesion, and maintaining the desired structural integrity and volume during healing (Drury and Mooney 2003). Drug-loaded hydrogels are often used to prevent the side effects of high-dose drugs and to increase local treatment efficiency (Mantha et al. 2019). Hydrogels can also be employed as encapsulation and stabilization agents for such bioactive molecules as growth factors, secretory cells, and stem cells in addition to drug loading (Lee and Shin 2007; Koehler et al. 2018). The collagen/gelatin sponge that encapsulates a basic fibroblast growth factor for sustained release to promote wound healing (Kanda et al. 2014) and treatment of chronic skin ulcers (Morimoto et al. 2015) is a good example of this type of application. Another is the hydrogel developed by Wang et al. (2008) in the form of a chitosan cross-linked collagen sponge containing recombinant human acidic fibroblast growth factor to increase diabetic wound healing. The review of Singh et al. (2016) provides an extensive listing of studies on the delivery of various bioactive materials using hydrogel scaffolds.

Both synthetic and natural materials are used to create hydrogels in tissue engineering. Polyethylene oxide, polyvinyl alcohol, and polyacrylic acid are examples of commonly used synthetic materials (Naahidi et al. 2017). Agarose, alginate, chitosan, collagen, fibrin, gelatin, pectin, lignin, and hyaluronic acid are naturally derived gel-forming biomaterials (Mohammadinejad et al. 2019; Kalai Selvan et al. 2020; Samadian et al. 2020). Using marine-derived biomaterials together with synthetic/natural materials or bioactive molecules, several hydrogel scaffolds have been developed for different applications. Some examples are chitosan/sodium alginate (Wang et al. 2017b), chitosan/hydroxyapatite (Nawrotek et al. 2016) and collagen (Hsiao et al. 2015) hydrogels for the nervous system; alginate (Valentin et al. 2017), chitosan/gold nanoparticle (Nezhad-Mokhtari et al. 2020), and hydroxyapatite-alginate-chitosan-fucoidan (Sumayya and Kurup 2017) hydrogels for tissue engineering applications; and collagen/alginate (Hahn et al. 2006), collagen/PEG/hyaluronic acid (Hardy et al. 2015), collagen/alginate/nano-hydroxyapatite (Zheng et al. 2014), chitosan/hyaluronic acid (Miranda et al. 2016), fucoidan/hyaluronic acid/gelatin (Lu et al. 2019) hydrogels for the vocal cord, soft tissue, osteochondral tissue, periodontal tissue, cartilage repair applications, respectively. However, hydrogels suffer from insufficient mechanical properties due to high water content, irregular spreading of cross-linking per unit volume, and varying polymer chain lengths (Costa and Mano 2015). For example, alginate hydrogels, which have been developed for cartilage repair and regeneration (Rubert et al. 2012; Farokhi et al. 2020), have limited use due to unfavorable mechanical properties (Igarashi et al. 2010).

Porous Scaffolds and Foams: The three-dimensional polymeric or ceramic porous scaffolds or foams with interconnected pore networks are excellent environments for host tissue or bone growth or organ vascularization in tissue engineering applications (Zhang and Ma 1999). The network of pores in the solid structure of a porous matrix or a foam offers an environment for a very effective adhesion and proliferation area for cells by:

- Providing large physical surfaces for cells to lay their own ECM,
- Inhibiting cell growth of adherent contact-inhibited cells,
- Improving nutrient transport through the interconnecting channel network, and
- Limiting cluster size and eliminating very large clusters that can potentially develop a necrotic center (Dhandayuthapani et al. 2011).

Polymeric porous or foam scaffolds are mainly manufactured by the thermally induced phase separation or one of the gas, liquid, or solid templating methods or the described above. Though the pore size and interconnectivity control are limited, these methods are the most promising, flexible, and tunable route for the preparation of 3D interconnected polymeric porous structures. Depending on the choice of solvent and phase separating conditions, the foams can be controlled to form either random or oriented pore architectures (Ma and Zhang 2001).

Among the marine biomaterials, alginate attracts attention in the foam production due to its flexibility and pliability that preserves the structural integrity, tensile

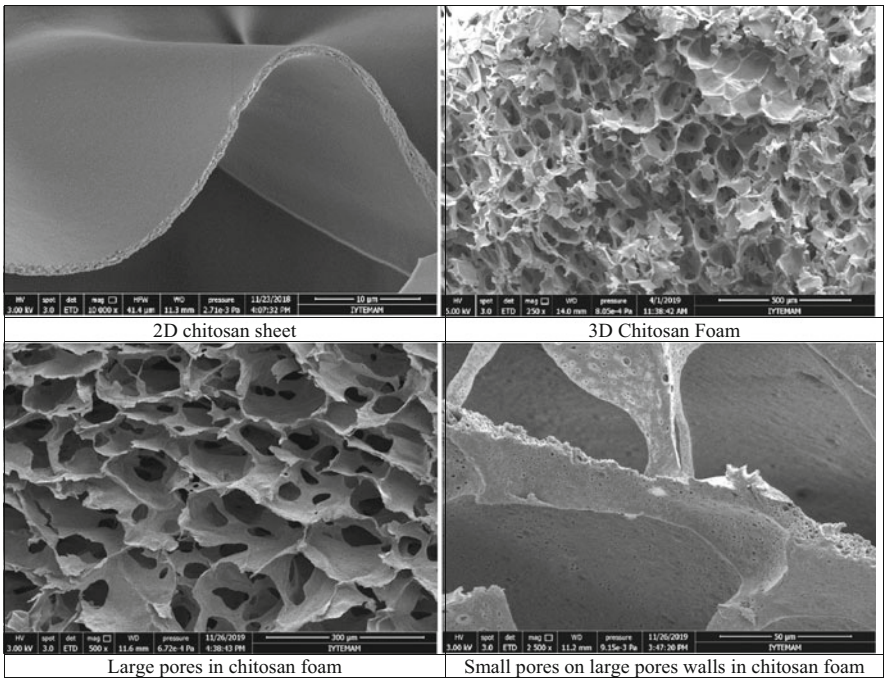

Fig. 7.3 Chitosan sheets and foams

strength, nonfragility in processing, and ease of sterilization (Guarino and Ambrosio 2014; Catanzano et al. 2018). Among the different techniques, freeze-drying is the most used technique to produce alginate foams (Ceccaldi et al. 2017). At the same time, alginate foams are used as a matrix that can trap drugs, cells, or bioactive substances in their pores. Alginate has been also used as an injectable gel in its pure form and in a blend with chitosan for bone tissue (Park et al. 2005; Li et al. 2005) and cardiac tissue (Radhakrishnan et al. 2014) as an example.

Polat and Polat (2020) and Polat et al. (2020a) produced 2D chitosan sheets and 3D chitosan foams using the emulsion polymerization method by the addition of a negatively charged cross-linking agent (Fig. 7.3). They were able to merge the gas and liquid templating methods in the same system, which yielded a double pore structure. The foam displayed very large pores with sizes in the order of 100 micrometers whose walls contained a much smaller pores with sizes in the order of 10 nanometers. The foams manufactured displayed very high compressive strengths (up to 250 kPa) and high prosities (up to 10 m^2/gram) compared to the other chitosan-based scaffolds produced in the literature (Xu et al. 2017). The authors were able to incorporate a low molecular weight hydrophilic model antibiotic vancomycin hydrochloride in the nanopores of the chitosan matrix walls. They found that the drug release mechanism of the chitosan foams depended on the conditions used for foam development. The released amount of vancomycin was

found to be higher at pH 5.6 than pH 8.2 due to the higher solubility of chitosan under acidic conditions suggesting that the drug release was proceeding in parallel with foam (chitosan dissolution).

Inorganic porous biomaterials, like hydroxyapatite, are also commonly used for bone tissue engineering applications because of their bioactivity, in particular for bone-tissue-related applications (Ahmed et al. 2019; Kumar et al. 2011; Bose et al. 2012), but they could also find other applications in contact with soft tissues (Miguez-Pacheco et al. 2015). The use of bioactive glasses is also valuable for tissue engineering applications because of their angiogenic, osteogenic, and antibacterial properties (Hoppe et al. 2013; Jones et al. 2006). Despite these properties, these inorganic biomaterials showed also low mechanical strength and low fracture toughness, limiting their applications in load-bearing tissue regeneration (Gerhardt and Boccaccini 2010). Inorganic porous scaffolds can be manufactured by incorporating the inorganic particles in a polymeric matrix and subsequently process it to fabricate porous composite scaffolds by using electrospinning or thermally induced phase separation (Liverani et al. 2019).

7.5 Loading of Marine-Based Scaffolds with Bioactive Molecules

The aims of delivery systems are to protect the bioactive molecules until they reach and are released at the targeted tissue and to keep them at optimal therapeutic levels in the body during treatment. Polymeric scaffolds, which are specifically planted at the intended site, are ideal delivery systems for targeted and controlled release (Ardeshirzadeh et al. 2015; Calori et al. 2020). Drug or bioactive molecule-loaded scaffold materials in the form of foams, sheets, or films are excellent antimicrobials, antifungal, anti-inflammatory, and tissue repair vehicles. Hence, it is very important to provide tissue therapy by taking advantage of the synergistic effects of drug delivery and the scaffolding system.

Bioactive molecules can be loaded into polymeric scaffolds in different ways. The suitable method should be chosen or designed according to the structure of the scaffold and the active molecule, the desired release rate, and the environment to be applied. The common methods for loading the scaffolds with active molecules are listed below.

Blend loading: Blend loading is one of the most common methods where polymers and active molecules are blended before fabrication (Calori et al. 2020). However, the interactions of the polymer and the active molecules should be considered. Except for specific binding or molecule–polymer conjugation, unsuitable physical/chemical interactions between the active molecules and the polymer cause phase separation, heterogeneous distribution and aggregation of the active molecules on the surface, and crystal structure (Seif et al. 2015; Perumal et al. 2008). The dissolution of the polymer and active molecules in the same solvent can also be

problematic, similar to the difficulties encountered when dissolving a water-soluble active ingredient together with a hydrophobic polymer.

Soak loading: In this method, the scaffold is prefabricated and then dipped in a solution containing the active molecules. Similar to blend loading, matrix wettability, pore size, and molecule–polymer interactions play a role in loading efficiency (Sriamornsak et al. 2010; Zhu and Mao 2019).

Site-specific binding loading: Unfavorable solvent or molecule–polymer interaction problems can be solved by covalent binding of the active molecules to the polymer or by site-specific binding of the active molecules through surface functional groups.

Encapsulation loading: In this approach, the active molecules may be encapsulated in nano- or microparticles before its introduction into the polymer solution or the scaffold material to prevent unfavorable molecule–polymer interactions. This method may allow more favorable molecule–polymer interactions by modifying the particle surfaces or more controllable release by modifying the structure and chemistry of the encapsulating particles (Calori et al. 2020; Polat et al. 2020b; Cihan et al. 2017; Çevik Eren et al. 2019; Zeybek et al. 2019; Polat et al. 2017; Sop et al. 2017).

The details of these methods and their applications can be found in an excellent review by Calori et al. (2020). In literature, there are several types of drugs and many different synthetic/natural polymers used for these kinds of applications. For example, chitosan/collagen/poly(N, N′-dimethylacrylamide) composite scaffold (Barroso et al. 2014); chitosan-based hydrogel (Yang et al. 2017); polycaprolactone/chitosan (Shi et al. 2020); and chitosan/gelatin films (Tejada et al. 2017) were loaded with ibuprofen, taxol, meloxicam–mitomycin-C, and miconazole by blend method, respectively. Mesoporous microspheres of carbonate-substituted hydroxyapatite (prepared by abalone shells) were used as a carrier for doxorubicin in a drug delivery system by using soaking loading (Huang et al. 2020). Hematite-doped bioglass/chitosan scaffolds were developed as a multifunctional alternative implant system for delivering chlorhexidine gluconate for endodontic therapy (El-Sayed et al. 2020). A summary of biomaterial scaffold-based local drug delivery systems for cancer immunotherapy can be found in a review by Yang et al. (2020).

7.6 Controlled Release of Bioactive Molecules From Scaffolds

The objective of the controlled release of bioactive molecules is to keep the drug concentration in the blood between the therapeutically minimum effective and minimum toxicity concentration and to provide effective treatment without causing side effects. Many studies have been conducted to design scaffolds as drug delivery platforms capable of controlled and sustained release of therapeutic agents (Rai et al. 2005; Hu et al. 2014; Calafi et al. 2014; Zhang et al. 2017b; Li et al. 2017; Ong et al. 2018; Kim et al. 2004; Álvarez et al. 2020; Wei et al. 2020; Obayemi et al. 2020;

Mandal and Kundu 2009). The release mechanisms, mathematical models, and related polymers are summarized in a review paper by Moradi Kashkooli et al. (2020).

The factors affecting the release of bioactive molecules from scaffolds are quite diverse and there are many approaches used to modulate them. The release rate can be controlled by:

- Structural features and physicochemical properties of the scaffolds,
- Chemical structures of drugs,
- Active molecule–scaffold interactions,
- The environment into which the release will take place.

The drug-loaded scaffolding should be designed for considering the release kinetics. For example, if drug molecules are placed in the core of nonbiodegradable scaffolds they may not be desorbable, leading to low release rates. Low release rates will also be the case if the scaffolds do not have sufficient porosity (Zupančič et al. 2015). In the case of hydrogels, the mesh size that regulates the diffusion of active ingredients in hydrogels is the critical parameter for release. The mesh size, which can be regulated by the polymer and cross-linking agent ratios during hydrogel production, should be compared against the size of the molecules to be released. Another important issue is the physical and chemical interactions between the polymers (matrix) and the active molecules. The rate of drug release can be achieved by accurate modulation of these parameters (Fig. 7.4).

As an example, the chitosan-graft-β-cyclodextrin composite scaffold was produced as a potential active filling material with controlled drug release that enhances tissue regeneration. It was shown that the drug release properties of the scaffold depend on the intensity of the cross-linking (Prabaharan and Jayakumar 2009). In another study, the release rate of poly (L-lactic acid)-chitosan hybrid scaffold produced has been controlled by chitosan content and the cross-linking densities (Prabaharan et al. 2007).

In addition, on-demand drug release can be performed by biological or external stimuli (Davoodi et al. 2018). There are polymeric substances such as chitosan, which respond to such stimuli. In addition, different approaches such as surface modifications of scaffolds, coating, forming composite structures with alternative sensitive materials, or the introduction of nanoparticles can give these polymers the ability to respond to stimuli. Light (attached with photosensitive functional groups like derivatives of azobenzene and nitrobenzene) (Vivero-Escoto et al. 2009), pH (Anna and Katarina 2018), reducing agent, temperature (Coughlan et al. 2004; Lindner et al. 2008), ultrasound, electrical (Pierce 2010), and magnetic fields (incorporating inorganic materials such as iron, cobalt, and nickel) (Pirmoradi et al. 2011) can be given as examples of internal or external triggers in this approach.

Another example release data has been obtained with a hydrophilic antibiotic drug (vancomycin hydrochloride as a model drug) loaded into chitosan foam by Polat et al. (2020a). They found that the drug release mechanism of the chitosan foams depended on the conditions used for foam development. The released amount

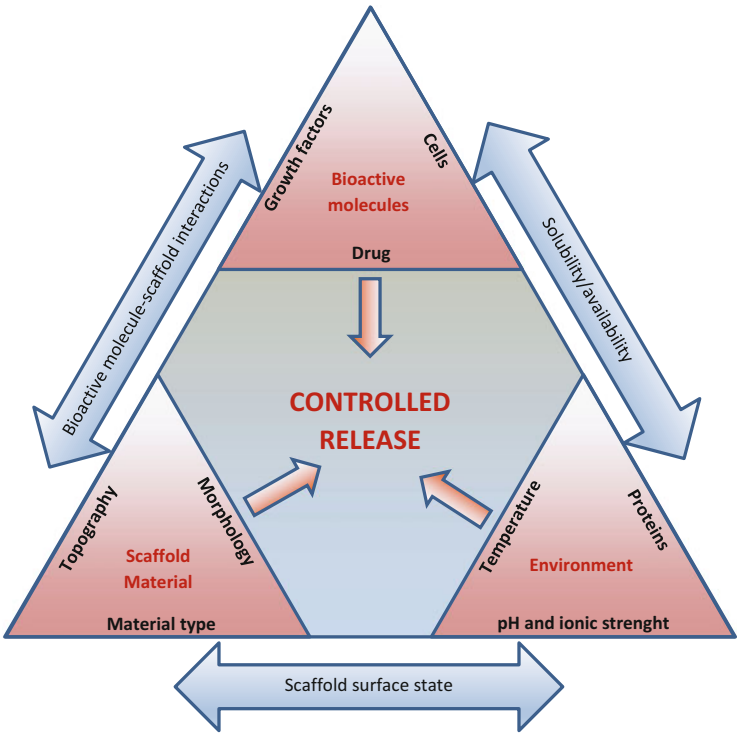

Fig. 7.4 Parameters that control the release of bioactive molecules

of vancomycin was found to be higher at pH 5.6 than pH 8.2 due to the higher solubility of chitosan under acidic conditions.

7.7 Physical and Chemical Characterization of Scaffold Systems

The important physical, chemical, and biological properties of solids, porous structures, fibers, or gels, which should be characterized as part of scaffold design, have been summarized in Fig. 7.5 along with the techniques, which can be employed exclusively for the study of a particular property. The purpose, operational details, and the analysis principles of each technique cannot be presented here for sake of brevity but can be easily obtained from the literature.

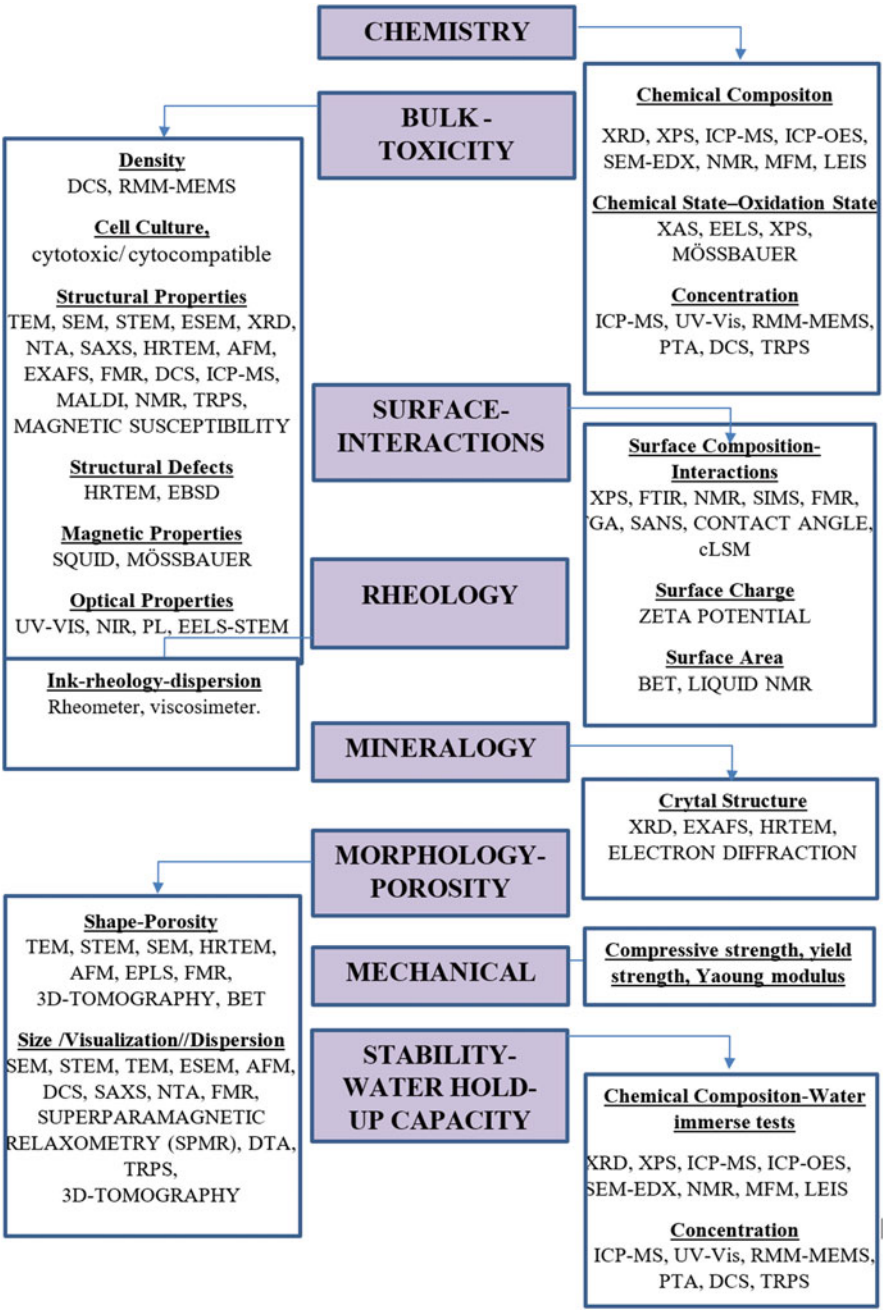

Fig. 7.5 Common methods which can be employed to characterize scaffolds (modified from Mourdikoudis et al. 2018; Polat and Polat 2020).

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Marine Biopolymer for Theranostic Applications

8

Gopal Kannan, Jaydeb Pal, Seshen Sivasankar, and Ayyavu Mahesh

Abstract

Recently, there is a wide synthesis of nanomaterial using marine-based biopolymers for the biomedical application because of their vital properties such as biodegradability and biocompatibility. The polymeric nanoparticles of high therapeutic efficacy play a major role in various types of cancer treatment including chemotherapeutics and theranostics. Notably, the polymeric nanoparticle helps to diagnose and treat numerous types of cancer where polymer provides good biocompatibility to therapeutic and theranostic agent; moreover, inorganic nanoparticle provides excellent absorption in NIR zone which is appropriate for clinical and biomedical application. Theranostics is the combined approach of therapeutic and diagnostic imaging techniques in which the drugs are delivered to the target site and NIR laser was used to penetrate the tissue. In the chapter, we have outlined the mechanism and efficacy of marine biopolymer-based nanomaterial as well as described both in vitro and in vivo studies of theranostic applications.

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Keywords

Marine biopolymer · Cancer therapy · Diagnostic · Inorganic nanoparticle · Theranostics

8.1 Introduction

Marine-based biopolymers are available in abundant and composed of long chains of repeating monomer units which are linked via glycosidic bonds. Especially, the fucoidan, alginate, carrageenan, and porphyran biopolymers were isolated from seaweed whereas chitosan and chitosan oligosaccharide (COS) biopolymers were derived from marine crustaceans (Manivasagan and Oh 2016). Marine biopolymers possess storage and structural functions and contribute to biological system including glycocalyx and extracellular matrices. Marine biopolymers are highly stable, more renewable, abundantly available, very cheap, good biodegradable, strong biocompatible, and nontoxic. In recent years, biopolymers are used in various fields including cosmeceuticals, nutraceuticals, and pharmaceuticals (Aider 2010). Until, more than 3000 new substances have been identified and isolated from marine organism among which few of them are commercialized and many compounds are under preclinical trials (Altmann 2017). Theranostics approach, helped to identify the characteristics of cancer such as cellular phenotype, tumor heterogeneity, and imaging for diagnostic purpose (Funkhouser 2002; Sumer and Gao 2008), where the high penetrating capacity of Near Infrared (NIR) lights is used to target blood and tissues (Muddineti et al. 2015).

8.1.1 Application of Theranostics in Cancer Treatment

Approximately, more than 250 types of cancer have been identified, among which prostate, breast, lung, colon, rectum, bronchus, and urinary bladder cancer are predominant (Wogan et al. 2004). Still, we have inefficient treatment method (radiotherapy and chemotherapy) with side effects and complication in receiving the timely diagnosis. The shortage of treatment option, emerging novel nanomedicine tends to offer new platform for biomedical application (Yang and Yu 2016; Kemp et al. 2016). The hydrophobic drugs are encapsulated with aqueous nanoparticle structure in order to deliver and release the drug to target site in cancer treatment (Kumar 2000). The comparative studies suggested the polymeric nanoparticles have a high potential of drug encapsulation, drug delivery, prolonged circulation, half-life, and sustained drug release than free drugs (Petros and DeSimone 2010; Wang et al. 2012). In active mode of targeting, the polymeric nanoparticles are incorporated with desired target moieties such as receptor or cell surface ligand whereas in passive targeting, polymeric nanoparticle aggregates at the target site because of their Enhanced Permeability and Retention (EPR) (Farokhzad and Langer 2009; Timko et al. 2011; Fang et al. 2013).

The various phases of clinical trials are carried out with 35,000 biological samples and dozens of candidates to treat the different type of cancers. (Haefner 2003). Theranostics have shown potential application in cancer treatment especially photoablation therapy, hyperthermia therapy, photoacoustic imaging (PAI), magnetic resonance imaging (MRI), and computed tomography (CT) (Mondal et al. 2013).

8.1.2 Polymeric Nanoparticle-Based Theranostic Material

Marine biopolymers are recognized as a flexible biomaterial and possess good biocompatibility, biodegradability, inexpensive, abundance, ease of surface modification, and nontoxic in nature (Sundar et al. 2010). In addition, the inorganic nanoparticles (NPs) including gold NPs (AuNPs), silver NPs (AgNPs), magnetic NPs (MNPs), mesoporous silica NPs (MSNs), copper sulfide NPs (CuSNPs), and upconversion NPs (UCNPs) are confirmed to exhibit strong scattering and absorption of light in NIR zone (700–1100 nm), which serves as good transparency media in biological tissue and blood (Wang et al. 2014). The cytotoxic nature of synthesized inorganic NPs is the major drawback for direct clinical application; hence few surface modifications and nontoxic polymer coating are required for enclosing the NPs surface to enhance biocompatibility (Dickerson et al. 2008; Liu et al. 2014).

The polymeric nanoparticle consists of three main components which are (1) polymers—to provide stabilization and biocompatibility, (2) therapeutic agent—may contain small molecule drugs, and (3) imaging agent such as MRI contrast agent, radionuclide, fluorophore, etc. Some therapeutic compounds itself serve as the imaging component, e.g., doxorubicin emits inherent fluorescence (Mohan and Rapoport 2010). The polymer-conjugated drug molecules offer efficient drug loading capacity and prolonged drug release (Aryal et al. 2011).

8.2 Role of Marine Biopolymers in Photoablation Therapy

Photoablation therapy is classified into two types: Photothermal therapy (PTT) and Photodynamic therapy (PDT) which are used in surgery and chemotherapy (Kim et al. 2013). PTT is the method of photo-induced heat to kill the cancer cells (Wang et al. 2014) whereas PDT, used the Photosensitizer (PS) drug with visible light irradiation at certain wavelength, which triggers the reactive oxygen species (ROS) that destroy the cancer cells (Mondal et al. 2013).

8.2.1 Marine Biopolymers in Photothermal Therapy

PTT received more attention due to the minimum invasion, continuous recovery, less complication, and no damage to the adjacent tissue (Bao et al. 2016). The

hyperthermia (temperature) used in PTT treatment is whole body and localized hyperthermia, in which the localized hyperthermia has higher potential than whole body hyperthermia. In localized hyperthermia, the externally tunable control device produces the desired hyperthermic temperature (>43 °C) at target region to kill the cancer cells (Fig. 8.1). PTT requires high power laser to penetrate human cells and tissue in order to destroy cancer cells, which is considered as major drawback (Jabeen et al. 2014; Huang et al. 2011). Together with the immune-targeted Au, Ag and Cus were employed to generate heat-mediated destruction of cancer cells (Huang et al. 2011).

The photothermal efficiency of gold nanostructure when combined with chitosan performs as a best theranostic and therapeutic agent for anticancer treatment. The study suggested the combination of chemo and photothermal therapy could be the best therapeutic strategy ever. The NIR laser (808 nm) was used to target tumor sites with cisplatin hybrid nanosphere (CS-AuNR-Pt NSs), where the chitosan-conjugated gold nanorod is loaded with cisplatin to execute photothermal treatment. The hybrid nanocomposites target tumor sites, further externally produced localized hyperthermia temperature (49 °C) suppresses the multiple tumors and inhibits entire tumor growth by increasing the effectiveness of cisplatin (Chen et al. 2013).

In vitro cancer treatment revealed NIR-guided chitosan-coated Ag nanotriangle serves as good biocompatible photothermal transducers. In human embryonic Kidney cell (HEK), it exhibits cytotoxic activities whereas in human nonsmall lung cancer cells, it shows the dose- dependent activity (Boca et al. 2011).

The chitosan-conjugated nanocarrier is chemically cross-linked with pluronic F 68 and loaded with gold nanorocks (AuNRs) to develop functional nanocarriers with soft, flexible, and good reservoir characteristics for biological macromolecules and cancer therapy. In vivo studies of cancer treatment shown that the surrounding healthy cells and tissues were left undamaged, when NIR light operated with chitosan-conjugated gold nanorocks (AuNRs), which elucidates that AuNRs are suitable agents for photo-based therapeutic applications (Choi et al. 2011).

The reverse microemulsion (water in oil) approach to develop the polyethylene glycol modified chitosan (CG-PEG) NPs by using genipin as a linker to produce nanocarriers of indocyanine green (ICG) for PTT in mice. The CG-PEG-ICG NPs possess high in vitro photothermal toxicity as they wipe off 15% of cancer cell population when irradiated under in vitro whereas in vivo studies show the concentration higher than 100 mg/ml of CG-PEG-ICG NPs exhibited irreversible tissue damages which represent that NPs could be an excellent nanocarrier for PTT (Song et al. 2015).

The Fe_3O_4-P (St/MAA)-Chitosan-Au core/shell nanoparticles for dual imaging and photothermal therapy. The nanoparticle comprises the iron oxide, Poly styrene-co-methacrylic acid (P(St-MAA)), chitosan (CHI), and gold (Au). The core region of Fe_3O_4 is covered by gold nanoshell for MRI, CHI present in the middle layer for biocompatibility, and Au provides photothermal activity. Furthermore, the CHI is shown good biocompatibility in WST-1 assay of HepG2 and L929 cells (Wang et al. 2013).

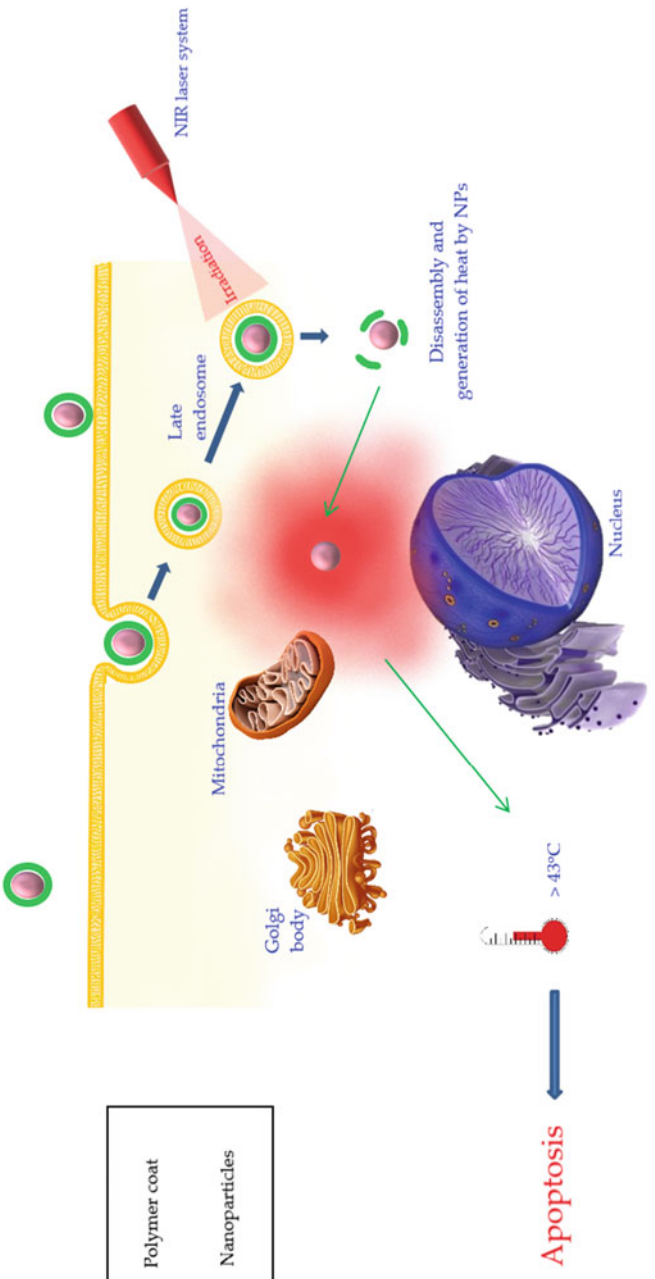

Fig. 8.1 Mechanism of cytotoxic effect of PTT-mediated cancer cell destruction. NIR radiation breaks the biopolymer coat and releases the NPs which initiate heat-mediated cell death by localized hyperthermic temperature 43 °C

Zhang et al. (2012) have been synthesized the AuNPs-coated chitosan nanocomposites through the reaction of HAuCl4 and sodium thiosulfate. Au NPs were coated with three different types of biopolymers such as chitosan (CS), O-carboxymethyl chitosan (CMCS), and the combination of CS and CMCS to develop the therapeutic agents for PTT. The in vitro studies of hepatocellular carcinoma (HepG2) and human dermal fibroblast cells indicate that the AuNPs-coated chitosan nanocomposites could be used as Photothermal agents because they exhibit stability, surface properties, low dosage, low power NIR light, continuous ablation, and photothermal effect on cell. Additionally, the CS- and CMCS-coated nanoparticles have a immense potential to differentiate the normal cells and cancer cells.

The HT-29 cells have shown the ease internalization of Folic acid-thiolated chitosan-modified gold nanorods (FA-CGNRs), when agitated with NIR laser. Furthermore, the FA-CGNRs act as excellent photothermal nano absorbers to target the desired region and kill cancer cells. The nanorods consist of thiolated chitosan to maintain the long stability and providing good biocompatibility to gold nanorod (AuNRs). The outer surface of chitosan-modified gold nanorods (CGNR) is connected by folic acid which links the amine group of chitosan and performs as a linker (Wang et al. 2011a, b).

The Doxorubicin (DOX)—conjugated chitosan-coated gold nanorod (DOX–CSGNR) provides more therapeutic efficiency than chitosan gold nanorod due to the good biocompatibility, short time of exposure, stability, and response to low intensity of light. In PTT, the DOX-CSGNR binds to the anticancer drug, and serves as an anticancer agent that expresses less cytotoxicity (Duan et al. 2014).

8.2.2 Marine Biopolymers in Photodynamic Therapeutic Role

Photodynamic therapy (PDT) is an excellent modern target therapy that has minimal invasiveness potential and produces cytotoxic effect on target cells and tissues. The photosensitizers (PS) were administered followed by the irradiation of the target site with corresponding wavelength to initiate photochemical reaction. The series of processes leads to the conversion of free oxygen and other free radicals to reactive oxygen species (ROS). The Reactive Singlet oxygen molecules ($^1O_2$) can cause the apoptosis and necrotic cell death in tumor tissue (Fig. 8.2). PDT can exclusively inhibit cancer by damaging the tumor vasculature, direct cytotoxic effects and induction of systemic immunity on tumor cells (Agostinis et al. 2011). Since, PDT offers less side effects or no toxicity to the adjacent tissues (Detty and Gibson 2004), the technique would be more specific than conventional therapies associated with adverse side effects. The properties of PS such as poor solubility in water, accumulation, and low reactive Oxygen Species (ROS) production, limit medical usage and so PS alternatives are being developed to improve efficacy (Bechet et al. 2008). The drawback of using PDT is photosensitizer gets aggregated in patient's body therefore making them light-sensitive after treatment (Huang et al. 2008).

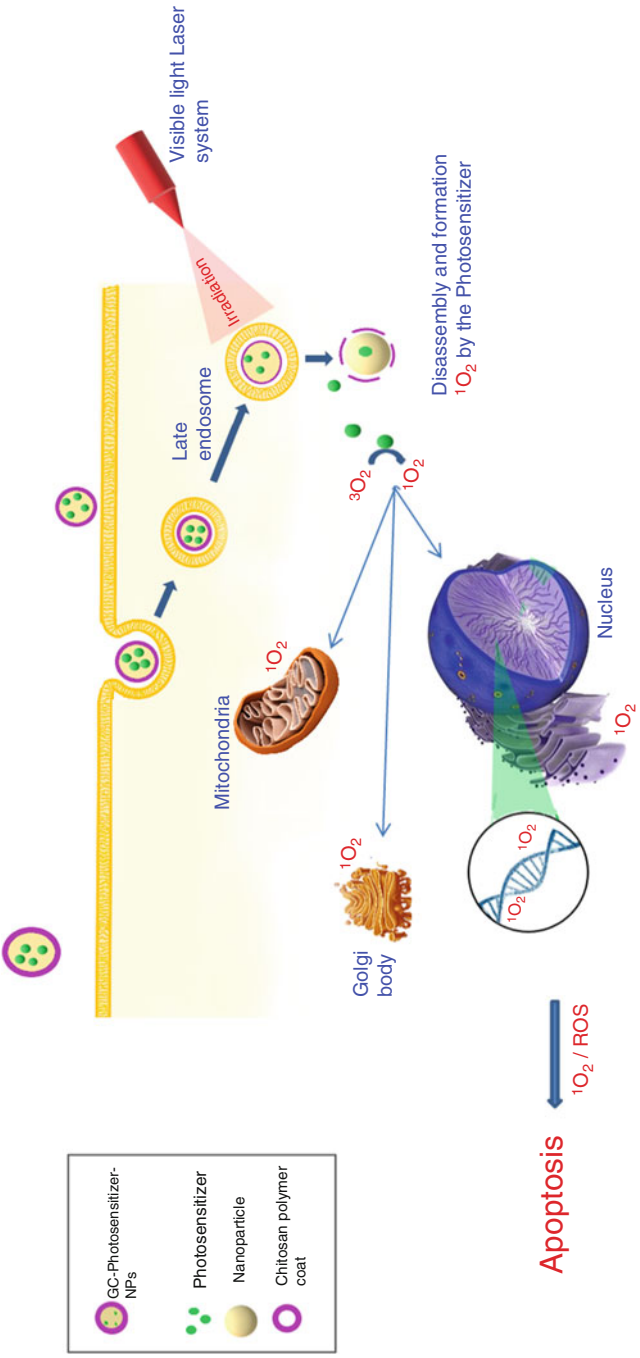

Fig. 8.2 Mechanism of cytotoxic effect of PDT-mediated cancer cell destruction. The Marine biopolymer coat breaks and releases the photosensitizer into the cell when irradiated followed by the ROS-mediated cell death by photosensitizer bound to NPs

The Protoporphyrin IX-conjugated glycol chitosan nanoparticles (PpIX-GC-Nps) are extensively used as drug carrier owing to the excellent tumor homing ability and cell-mediated on/off system in photodynamic therapy. The PpIX-GC-Nps are spherical nanostructures with average diameter of 280 nm. When cell encounters the PPI-GC—NPs, intracellular environment turns the nanoparticle "on," subsequently the therapeutic ability gets restored. Further, the nanoparticles are shown to reduce unintended cytotoxicity, increase the therapeutic efficiency. Moreover, the nanoparticle possesses tumor harboring ability, prolonged blood circulation, good in-vivo therapeutic efficacy on tumor, and found to have great applications in PDT (Lee et al. 2011a, b).

The magnetic chitosan nanoparticles are developed as drug carrier and imaging system for MRI scan, where Photosensitizer 2,7,12,18-tetramethyl-3,8-di-(1-propoxyethyl)-13,17-bis-(3-hydroxypropyl) porphyrin (PHPP) is conjugated for therapeutic purposes. The MTCNP-PHPP complex is used in MRI-monitored targeting of PDT for its excellent targeting and imaging ability. The nanoparticle is quasi-spherical shaped with average diameter ranges from 15.5 to 25.7 nm. MTCNP-PHPP has 5.08% of drug loading and 65.4% of drug encapsulation capacity which is required for an effective PDT. In vivo and in vitro studies revealed the polymer exhibited nontoxicity and high photodynamic efficiency at a range of 0–100 μM. The theranostic-mediated treatment, PHPP is eliminated by the body thereby after treatment photosensitivity was reduced in patients (Sun et al. 2009).

O-carboxymethylated chitosan upconversion nanocrystal NaYF4:Yb/Er is comodified with pyropheophorbide a (Ppa) and RGD peptide C (RGDyK) to yield the final nanoparticle (CMC-UCNP-Ppa-RGD). The nanoparticles show enhanced water solubility, biocompatibility, stability, and resistance to PS self-aggregation during drug delivery. UCNP-Ppa-RGDs can target integrin $\alpha_v\beta_3$ positive tumor cells selectively and induce cell death when irradiated with Near-Infrared laser Radiation (NIR). Moreover, the nanostructure shows great potential for targeted NIR-based PDT (Zhou et al. 2012). Similarly, Amphiphilic N-succinyl-N-octyl chitosan-modified Oleic Acid capped UCNPs (SOC-UCNP) are conjugated with PS like zinc (II) phthalocyanine (ZnPc). The SOC-associated UCNPs improve bio compatibility, reduced toxicity, and provide layer for carrying the hydrophobic drug molecules. The intracellular colocalization of the UCNPs and ZnPc can be ensured by the fluorescence of the UCNPs. Attachment of nanoparticles to Photosensitizer facilitates the activation of PS and establishes ROS-mediated cell death in presence of NIR light. ZnPc-SOC-UCNPs possess potential PDT property such as deep penetration into tissues (Cui et al. 2012).

The folic acid-conjugated chitosan-based nanoparticles were integrated with alginate and loaded with 5-aminolevulinic acid (5-ALA). Remarkably, the nanoparticles loaded 5-aminolevulinic acid (5-ALA) binds selectively to the colorectal cancer cells via folate receptor-mediated endocytosis and shows great specificity toward colorectal cancer. The nanoparticles are internalized; 5-ALA molecules were released into the lysozyme because of less affinity between the NPs and 5-ALA due to the presence of deprotonated alginate where accumulation of protophyrin IX allows successful photodynamic detection (Yang et al. 2011).

Hydrophobic glycol chitosan (HGC) or glycol chitosan (GC)-conjugated chlorin e6 (photosensitizer) loaded nanoparticles were used for PDT, where the HGC polymers release Ce6 into the cells in a time-dependent manner. The HGC-Ce6-associated drug shows less aggregation in the tumor tissue and enhanced tumor target drug delivery, whereas GC-Ce6 possesses prolonged circulation, efficiently accumulated in the tumor tissue and shows excellent PDT potential than HGC-Ce6 (Lee et al. 2011a, b).

Pheophorbide A (PheoA), a glycol chitosan (GC) with reducible disulfide bonds (PheoA-ss-GC) bound with core–shell NPs (200 nm) was evaluated with nonreducible Pheo-GC. The photoactivity of both NPs is greatly inhibited by the quenching effect; however, the bio reducible NPs overcome the quenching effect by instant dissociation of the compound by the cleavage of the disulfide bond that is facilitated through the intracellular reductive nature. The rapid cellular uptake of reducible NPs and their high phototoxicity makes it more compatible for PDT than the nonreducible NPs. Further, in vivo studies showed the bioreducible NPs get accumulated specifically in tumor tissue and have prolonged circulation in the blood (Oh et al. 2013).

8.3 Marine Biopolymers in Hyperthermia Therapy

Hyperthermia therapy (HTT) is an excellent method to target specific tissues, exclusively used to monitor, and treat cancer patients. HT acts on tissues by increasing the temperature greater than 40 °C at specific sites; moreover, a temperature of 43°c causes enzyme denaturation, cell necrosis, and functional changes in DNA (Fig. 8.3). Furthermore, the hyperthermic condition breaches the cell membrane resulting in cell death (Westermann et al. 2005).

The combined action of Magnetic Nano Particles (MNPs) and Hyperthermia therapy is a novel strategy which allows controlled heating on the tumor tissue where MNPs reside in the target tissue, increases the local temperature by transforming the electromagnetic energy into heat by the oscillation of the external magnetic field, thereby helping to get rid of damages to the adjacent normal tissues (Lima-Tenorio et al. 2015).

The coprecipitation of Fe^{3+} and Fe^{2+} with aqueous NaOH solution yields Fe_3O_4-chitosan MNPs. The temperatures of Fe_3O_4 NPs and Fe_3O_4-Chitosan nanocomposites were found to be 95.5 and 53.7 °C when exposed to alternate current magnetic field for 20 min. The temperature produced by nanocomposites is good enough to destroy the cells and an excellent thermo seeds for the localized hyperthermia therapy (Zhao et al. 2009). Relatively, Fe_3O_4 MNPs could be significant agent for hyperthermia therapy. The nanoparticles are synthesized with alkaline precipitation of ferrous chloride and coated with chitosan by ultrasonification (Patil et al. 2014).

Alginate magnetic nanoparticle is synthesized by two step process: Initially magnetite was distributed in the alginate sodium solution by ultrasonification and homogenization process followed by reticulation with calcium ions. The yielded

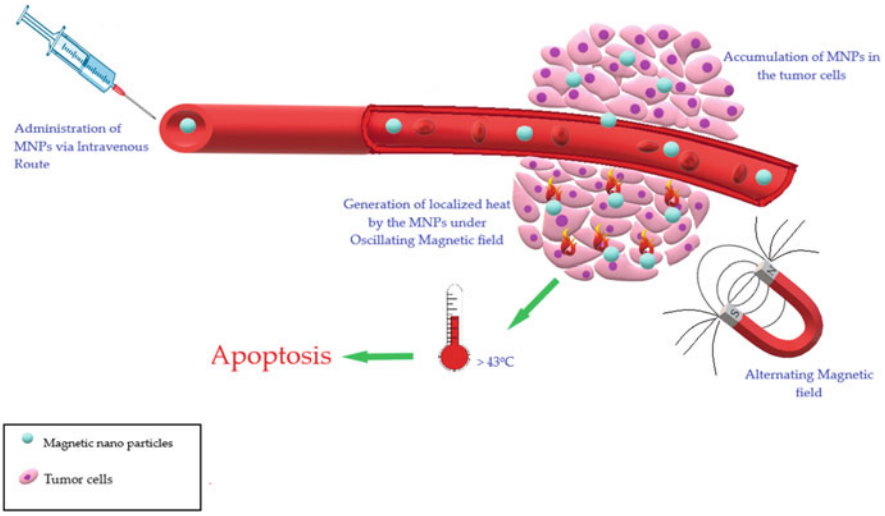

Fig. 8.3 Mechanism of HT-mediated destruction of cancer cells. Administered MNP selectively binds to the tumor cell and generates heat with the help of external AC magnetic field leading to the apoptosis/necrotic cell death

NPs are 400 nm in diameter, more stable, have good magnetic properties, capable of entrapping, and release of drug to the desired site due to external magnetic manipulation (Ciofani et al. 2009).

Chitosan oligosaccharide-stabilized ferrimagnetic iron oxide nanocubes (Chito-FIONs) are made up of multiple FIONs that are encapsulated in chitosan-L-3,4-dihydroxyphenylalanine (DOPA) polymeric shell. The nanoparticles are magnetically monitored to target the site and activated via remotely through external alternating current magnetic field to initiate cytotoxicity by increasing localized temperature and induce caspase-mediated apoptosis in certain type of tumor cells (Bae et al. 2012).

The Iron oxide (Fe_3O_4)-encapsulated chitosan NPs was cross-linked with Sodium tripolyphosphate (TPP) to obtain magnetic core–shell chitosan nanoparticles containing approximately 5.6% (w/w) of Magnetite. The 259 nm sized nanoparticle was found to increase the temperature when agitated by external alternating current magnetic field and considered to be good in hyperthermia-based therapy (Zamora-Mora et al. 2014). Similarly, the Fe_3O_4– chitosan NPs produced by facile method showed the magnetic property and excellent hyperthermic property (Qu et al. 2010). Starch-coated MNPs and chitosan-coated MNPs were analyzed for their cytotoxicity in L929 cells, where chitosan-coated MNPs were capable of generating 23 °C more than the starch-coated MNPs under AC magnetic field. Moreover, chitosan plays an important role in cell adhesion of hyperthermic thermo seeds and shown biocompatibility of the magnetic nanoparticles. Chitosan-coated nanoparticles are expected to be more suitable polymer for wide range of cancer therapies (Kim et al. 2009).

Li et al. (2018) have been developed the PEGylated indocyanine green (ICG)—loaded Polypyrole Nanoparticles (PPI NPs) and used the polydopamine as a linker. The in vitro and in vivo assay proved PPI NPs were internalized in Hela cells and retained in the tumor due to its biocompatibility which was also confirmed by in vitro *flow* cytometry/confocal studies and in vivo photoacoustic imaging. The photoacoustic imaging revealed the photothermal destruction of tumor was achieved when PPI NPs are operated with laser. PPI NPs offer high photothermal, photoacoustic effect where PPI NPs play a main role by increasing the retention time and accumulation at tumor site efficiently.

Chitosan-polypyrrole nanocomposites (CS-PPY NCs) were used as novel agents for photoacoustic imaging-guided photothermal destruction of cancer cells, because NCs shown high stability, conductivity, biocompatibility, and extremely high absorbance in the near-infrared region (NIR). In vivo studies revealed the mice-bearing tumor cells are completely recovered when CS-PPy NCs are irradiated with (NIR 808-nm) laser (Manivasagan et al. 2017).

8.4 Marine Polymer Role in Photoacoustic Imaging

Photoacoustic imaging (PAI) is also known as optoacoustic imaging, which enhances the ultrasound with high optical contrast and can be used as an inexpensive portable instrument for visualizing blood vessels and body organs. PAI has major advantages including high spatial resolution, high imaging depth (Wang 2009); moreover, PAI has immense potential to image the endogenous chromophores such as oxyhemoglobin (HbO_2), deoxyhemoglobin (Hb), melanin, lipids, and water (both free and bound state). Besides, PAI can also image exogeneous chromophores—indocyanine green (ICG), methylene blue dye (MBD), nanoparticles, and reporter gene agents (Ntziachristos and Razansky 2010; Jacques 2013; Wu et al. 2014; Weber et al. 2016; Brunker et al. 2017).

Since smaller dosage of doxorubicin provides better effectiveness, doxorubicin-fucoidan- conjugated gold nanoparticles (DOX-Fu AuNPs) used as a contrast agent for PAI-mediated cancer therapy. DOX-Fu AuNPs facilitate the in vitro noninvasive detection and diagnosis of cancerous cells when irradiated with visible light laser system (532 nm) and the NPs treated MDA-MB-231 cells showed high amplitude of photoacoustic signal (Manivasagan et al. 2016). Paclitaxel-loaded chitosan oligosaccharide gold nanoparticles have also been implemented as a novel contrast agent for photoacoustic imaging (Manivasagan et al. 2016).

8.5 Magnetic Resonance Imaging (MRI)

Iron oxide nanoparticles have been used as a tool for magnetic resonance imaging of stem cells (Mahmoudi et al. 2011). Biopolymers made from polysaccharides are used in synthesis of iron oxide nanoparticles to promote size distribution and prevention of clustering (Villaraza et al. 2010; Wang et al. 2011a, b; Somsook

et al. 2005; Bhattarai et al. 2007; Saboktakin et al. 2009; Tsai et al. 2010). The iron oxide nanoparticles-conjugated chitosan polyacrylic acid (CS-PAA) template offers super paramagnetic properties thereby used for enhancement of magnetic resonance contrast by altering proton relaxation in the tissue microenvironment (Feng et al. 2009). Chitosan is being used to develop CS-PAA templates for MRI due to its outstanding biodegradability, biocompatibility, and making it useful for a large number of applications (Chen et al. 2011).

8.6 Computed Tomography

The AuNPs eliminated the drawbacks of Iodine-based CT agents which cause renal toxicity and fast excretion (Krause 2002). Furthermore, AuNps offer high X-ray absorption coefficient, low toxicity, and slow clearance in the body (Hainfeld et al. 2006). Gold nanoparticles bounded glycol chitosan ~~are~~ used in CT for tumor imaging, where injected nanoparticles (GC–AuNPs) are accumulated in tumor tissue within 30 min. Further the three dimensional image can be created with CT to reveal the stages of tumor across different regions of the tissue (Sun et al. 2014).

8.6.1 Theranostic Application in Bacteriology

The streptomycin loaded chitosan-coated magnetic nanocomposite (Strep-CS-MNP) to evaluate the antimicrobial activity against both gram-positive and gram-negative microorganisms. Initially, the Fe^{2+} and Fe^{3+} iron salts are coprecipitated in alkali media to produce the magnetic nanoparticles (MNP) and the MNPs were covered with chitosan in order to synthesize CS-MNP and Streptomycin was coated on the surface of CS-MNPs. The magnetic nature of nanoparticle follows the direction of magnetic field which can be exploited to detect the presence of microorganisms therefore possessing antimicrobial and antituberculosis applications. The sensitivity of imaging techniques such as MRI can be enhanced by using MNPs, hence (Strept-CS-MNP) can be used to diagnose the presence of harmful microorganism and treat the various type of microbial and tuberculosis infections (El Zowalaty et al. 2015).

8.7 Conclusion

The marine biopolymer-based nanomaterial has high potential than free drugs and provided numerous theranostic applications in cancer. In theranostic, marine biopolymer-conjugated nanomaterial can target the cell via either active or passive mode. Marine biopolymer offers surface modification and nontoxic polymer coating of nanoparticle to enhance the biocompatibility. Furthermore, polymeric nanoparticle offers the drug encapsulation, drug delivery, prolonged circulation, half-life, and sustained drug release than free drugs. Several studies revealed the combined imaging and therapeutic technique. Theranostics has a high efficiency than other

therapeutics strategy due to the early detection and diagnosis of cancerous cells. The in vivo and in vitro studies suggested that the marine biopolymer-conjugated nanomaterials exhibit cytotoxic, photothermal toxicity, increase the therapeutic efficiency, and excellent biodegradability. Marine biopolymer-mediated theranostic treatment could be a better approach to reduce the side effect of life threatening treatment including chemotherapy, radiation therapy, and nucleic acid therapy.

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9 Marine Biomaterials as Carrier of Drugs/Biomolecules for Management of Bone Disorders

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Abstract

Orthopedic disorders in human are the major concerns for the surgeons and medical specialties to treat in effective way. Various disorders of bone, such as osteoarthritis, rheumatoid arthritis, osteoporosis, and bone cancer, arise with time. Bone defects and nonunions need over 1,000,000 bone repair procedures annually worldwide. Osteoporosis is one of the major bone disorders according to World Health Organization (WHO). Osteoporosis is also known as "silent disease" as it does not give any sign of increase in bone fragility. Similarly, over 15,000 new bone cancers are cropped up every year with 3–5% of childhood cancers and $\leq$1% of cancers in adults of localized osteosarcoma have an average 5-year survival of about 80% but those with metastatic disease have much worse outcomes. Osteomyelitis is a severe and challenging setback in bone surgery. To address the above situations, various antibiotics, anticancerous drugs, bone growth factors, and usual treatment packages have been used. Conventional therapy in solving these situations does not always yield satisfactory results due to many reasons including lack of understanding of the challenges related to these bone disorders, lack of traditional treatment options available, high cost related to

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long hospitalization, and potential side effects from current treatment options. Specific and targeted delivery of drugs is an upcoming and alternative option to ensure safe and efficacious treatment with such bone disorders.

Due to abundance availability and suitable properties, marine organisms show its potential in biomedical applications for the management of various diseases. Owing to various complications and futility related to systemic drug administrations, marine materials can be utilized and developed as carrier of drugs/biomolecules for management of bone disorders. The effectiveness of drug delivery system will depend on the suitable properties and characteristics of carrier materials. In this chapter, we will highlight the marine materials as potential drug delivery carriers for management of various bone disorders. This chapter will also provide an overview of presently used marine materials as bone graft substitute vis-à-vis as carriers in local drug delivery systems.

9.1 Introduction

There are various affections of bone where foot and ankle arthrodesis, spinal fusion, nonunion, and tumor resection where revision surgery, bone void filling, stimulation of fracture healing, and bone grafting (Clarke et al. 2011) are essential. Though autograft and allografts are considered as gold standard in tissue engineering, they have some drawbacks too. In autografting, there is chance of pain, availability of less amount of graft material, and donor site morbidity, especially in elderly patients (Nandi et al. 2013). Whereas allograft is limited in stock (Galea et al. 1998), there are 20–30% chances of graft failure and immunogenic reaction (Louisia et al. 1999) and chances of disease transmission as well. In recent years, the bone grafts that are used clinically and experimentally are mostly synthetic and are composed of (CaP) ceramics. Bone comprises organic part, mainly collagen and other proteins and inorganic part composed of hydroxyapatite (HA) and other components similar to CaP, like beta-tricalcium phosphate. Hence, these materials are chosen for scaffold preparation or as bone substitute (Rezwan et al. 2006). Surprisingly, this mineral composition also prevails in marine environment where they provide mechanical and functional support to the marine organisms (Clarke and Walsh 2014).

On the other hand, biomaterials can be defined as the material, which has unique characters, which make them compatible to be used in living tissue and can mimic biological processes (Park and Lakes 2007). Selection of biomaterials is based upon certain criteria such as they should be biocompatible, bioresorbable, or biodurable, easily sterilizable, and good functional properties for which they are applied for (Ehrlich 2015).

Marine organisms own most of these characters and prove their potentiality to be used as ideal biomaterial. Ocean contains a variety of structurally exclusive natural products that are mainly assembled in the living objects. The fascinating beauty of

ocean, its biodiversity, and its inhabitants attract the human race through the ages. The marine ecosystem contains over 80% of world's plant and animal species having a wide thermal range (below freezing temperature in Antarctic water to about 350A°C in deep hydrothermal vents), pressure range (1–1000 atm), and nutrient range (oligotrophic to eutrophic) along with extensive photic and nonphotic zones. The enormous biodiversity in the marine setting far exceed that of terrestrial environment, and many potent biomedical compounds have been published recently from marine animals and other organisms (Donia and Hamann 2003).

The purpose of bone repair in various orthopedic problems can also be accomplished with the use of marine organisms as (i) they are capable to produce various forms of mineral ranging from submicron silica frustules to the aragonite skeletons, respectively, by diatoms to lobster and cuttlefish (Clarke and Walsh 2014). These minerals are found mostly in silica and calcium carbonate-based, in calcite and aragonite form. They provide scaffold that facilitates osteoconduction either ex vivo or in situ as they are capable to produce mineral in alkaline aqueous media even if all are not capable to utilize CaP of mammalian bone. (ii) Porous nature of marine organism is also an unique character, which makes them suitable candidate for orthopedic applications. Their pore size ranges from submicron to millimeter that facilitates their application in various tailor-made products, and help in bone in-growth, neovascularization, and resorption whenever they are necessary for specific purpose. Pores in marine organisms are interconnected and tortuous in nature, which provides them unique structural properties, which are hard to manufacture synthetically (Sharma and Elisseeff 2004). Due to these pore characters, they are potentially good bone substitute and serve as template for implant production. (iii) There are some ions such as carbonate (CO_3^{2-}), magnesium (Mg^{2+}), and silicate (SiO_4^{4-}), which were previously considered as impurities in CaP, and now several researches are found, which prove their importance in bone repair, osteoblastic adhesion, neovascularization, and bioresorption (Fellah et al. 2008; Coathup et al. 2011; Dorozhkin 2012; Holzapfel et al. 2013). Therefore, this ion substitution is considered to be beneficial with base ceramic considering the abundance of ions in marine organisms. Several researches have been carried out in last three decades for soft and hard tissue engineering emphasizing marine-based products to develop improved properties and design of implants, development of drugs or molecules, and drug delivery system. Most of the studies were carried out with marine proteins and biopolymers for this purpose.

9.1.1 Coral

First use of stony coral belonging to phylum Cnidaria was planned by White in the early 1970s (White et al. 1972) for bone repair and its clinical use started in 1980s (Vago 2008) (Fig. 9.1). It primarily acts as CaP substitute, but calcium carbonate, present in coral, also acts as osteoconductive and osteoinductive in nature. Several in vitro studies depicted that coral enhances osteoblastic and osteoclastic augmentation (Ehrlich et al. 2006) and, simultaneously, helps in propagation of mouse-derived

Fig. 9.1 Photograph showing sea coral

mesenchymal stem cell (MSC) line either as demineralized matrix (Ehrlich et al. 2006), in combination with chitosan or alone, with different species like *Porites lutea* (Gravel et al. 2006; Abramovitch-Gottlib et al. 2006; Tran et al. 2011) and *Millepora dichotoma* (Abramovitch-Gottlib et al. 2006). Corals used for scaffold preparation have pore size of 100 μm and 500 μm diameter mostly with excellent interconnected pores (Green et al. 2002; Abramovitch-Gottlib et al. 2006; Mygind et al. 2007; Geiger et al. 2007). Clinical and experimental applications of coral scaffolds either directly or as derivatives have been reported in various orthopedic problems like spinal fusion (Im et al. 2005; Coughlin et al. 2006; Griesshaber et al. 2007), dental surgery (Martina et al. 2005; Lahaye and Robic 2007; Julia et al. 2016), maxillofacial surgery (Oliveira et al. 2007; Laza et al. 2007; Chen et al. 2008), reconstruction of bone defects in rabbit model (Duy Huynh et al. 2017), sheep model (Decambron et al. 2017), canine model (Cui et al. 2007; Yuan et al. 2009; Liu et al. 2013) and in other orthopedic complications (Coughlin et al. 2006; Bächle et al. 2006; Hou et al. 2007).

9.1.2 Collagen

Collagen protein in abundance is found in connective tissue of mammals, derived from precollagen and extensively studied biomaterial. Bovine-derived collagen is mostly used, but they sometimes cause immunogenic reaction and there are chances of zoonotic disease transmission. Marine sources of collagen are safer option as compared to bovine collagen that derived from marine sources like jellyfish like *Stomolophus nomurai meleagris* (Song et al. 2006), *Cyanea nozakii* Kishinouye (Zhang et al. 2014), *Nemopilema nomurai* (Morishige et al. 2011), and sponge *Chondrosia reniformis* (Heinemann et al. 2007a). Hydrogels that have porous structure help in cell adhesion are obtained from collagen derived from jellyfish and sponge from the said species. It is reported that porous scaffold derived from jellyfish helps in proliferation of human mesenchymal stem cells (hMSCs) and

expression of chondrogenic markers as well (Hoyer et al. 2014). Marine collagen composite with chitosan and hydroxyapatite showed better proliferation of MG-63 cells than chitosan in pure form; thus, it was confirmed that marine collagen has positive therapeutic role in bone repair in various orthopedic and biomedical applications (Lin et al. 2011; Pallela et al. 2012; Subhan et al. 2015; Bayon et al. 2015).

9.1.3 Chitin and Chitosan

First isolation of chitin was reported from mushroom in 1811 (Braconnot 1881). Chitins are abundantly found in marine crustacean shell, especially from shrimps and crabs. Deacylated form of chitin is known as chitosan. Chitin and chitosan both have unique biological characters like they are nontoxic to body, biocompatible, and biodegradable and can easily be combined with polyvinyl alcohol, polyethylene glycol, or collagen which help to enhance their structural strength and cell adhesion property (Srivastava et al. 2015). They are extensively studied either alone or in composite with sponge (Seol et al. 2004), nanohydroxyapatite (Kong et al. 2006; Thein-Han and Misra 2009), calcium–phosphate (Zhang et al. 2003; Aryaei et al. 2015), alginate (Li et al. 2005) as bone graft substitute or scaffold (Costa-Pinto et al. 2011; Levengood and Zhang 2014), in repair of articular cartilage (Mattioli-Belmonte et al. 1999; Francis Suh and Matthew 2000; Malafaya and Reis 2009; Neves et al. 2011), preparation of injectable CaP cement (Moreau and Xu 2009; Weir and Xu 2010; Shavandi et al. 2016), and carrier of drug delivery system (Mattioli-Belmonte et al. 1999; Bhattarai et al. 2010; Patel et al. 2010; Elgadir et al. 2015; Ali and Ahmed 2018; Hamedi et al. 2018). Chitin and chitosan have other well-established role including enhancement of wound healing (Nishimura 2001; Değim et al. 2002), immune modulation, hemostatic activity, (Millner et al. 2009; Lan et al. 2015) and hypolipidemic activity (Zhang et al. 2011, 2012, 2013), and antimicrobial activity (Tsai et al. 2002; Zheng and Zhu 2003; Devlieghere et al. 2004; Goy et al. 2009). In gene delivery (Lu et al. 2014), cell culture and tissue engineering along with polymer chitosan are also used (Kurita 2006). Glucosamine, derived from chitosan, is clinically well-accepted molecule in treatment of osteoarthritis (Qian et al. 2013; Srivastava et al. 2015; Nagaoka et al. 2019).

9.1.4 Chondroitin Sulfate (CS) and Hyaluronic acid (HA)

Glycosaminoglycans (GAGs) are polysaccharide in nature made up of covalently linked disaccharide units and found in most of the mammalian tissue, and chondroitin sulfate (CS) is most important GAG found in whale, shark, rabbitfish, skate, squid, salmon, king crab, and sea cucumber (Im et al. 2009; Ustyuzhanina et al. 2017; Vázquez et al. 2018, 2019). It has also been isolated from marine invertebrates like Cnidaria, Polychaeta, and Mollusca (Volpi et al. 1998; Medeiros et al. 2000; Yamada et al. 2007; Adamczyk et al. 2010). CS helps in maintaining the elasticity of

articular cartilage, controls inflammation, and helps in hemostasis in addition to cellular differentiation, proliferation, and adhesion (Schiraldi et al. 2010). It is used to treat osteoarthritis (Clegg et al. 2006; Bruyere and Reginster 2007; Lee et al. 2009) as it possesses anti-inflammatory property (Peng et al. 2006; Iovu et al. 2008; Egea et al. 2010; Mou et al. 2018). It helps to hydrate the tissue due to the presence of high surface negativity (Henrotin et al. 2010). It takes important role in tissue engineering, neuroprotection, and wound healing (Gilbert et al. 2004). Hence, CS proved to be very potential candidate for tissue engineering and various clinical applications (Abdallah et al. 2020).

Hyaluronic acid (HA) or hyaluronan, a naturally occurring nonsulfated glycosaminoglycan in nature, is found in mammalian and marine organisms. It also helps in tissue hydration like chondroitin sulfate and controls the permeability of macromolecules between cells and bacteria (Constantinides et al. 2004; Anisha et al. 2013; Abdallah et al. 2020), hence acts as antimicrobial agent. Large numbers of water molecules are present in its molecular structure; hence, it can swell and help in lubrication of joint, and due to its unique chemical nature like viscoelasticity, biocompatibility, angiogenicity, and immune-stimulatory role, it is a good subject for tissue engineering and clinical applications. Due to lubrication property, it act as shock absorber in joints and help in movement and pain alleviation in osteoarthritis and rheumatoid arthritis (Roth et al. 2005; Sadozai et al. 2007; Anitua et al. 2007; Teeple et al. 2011; Shin et al. 2014). Several studies depict that nanocomposite of hyaluronic acid and chitosan have potential role in wound dressing, as it has antibacterial property (Abdel-Mohsen et al. 2013; Abdel-Rahman et al. 2016; Abdelrahman et al. 2020). It has important role in orthopedic applications as antimicrobial coating of implants (Disegi et al. 2005), together with platelet-rich plasma for enhancement of fracture healing (Martins Shimojo et al. 2019).

9.1.5 Alginate

Alginates are most common biopolymer derived from marine source mainly from brown seaweeds species of *Laminaria*, *Macrocystis*, *Lessonia*, *Ascophyllum, Ecklonia, Durvillea,* and *Sargassum* and industrially manufactured (Gomez et al. 2009; Venkatesan et al. 2014b). The properties like biodegradability, controlled porosity, non-toxicity, and anti-inflammatory action have enabled this product to be used in various therapeutic and research purpose. It is used to produce scaffold for orthopedic application as alginate is readily cross-linked with calcium ions (Srivastava et al. 2015). But, to overcome its inherent drawbacks like low mechanical strength and less cellular adhesion commonly, it is combined with other materials. Studies depicted that incorporation of into calcium carbonates into alginate enhances mineralization of extracellular matrix and helps to differentiate mesenchymal stem cells into osteoblast (Diaz-Rodriguez et al. 2018). In vitro study showed polymer scaffold prepared with chitosan–alginate–fucoidan (Chi-Alg-fucoidan) and chitosan–alginate–single-walled carbon nanotubes (Chi-Alg-SWNT) scaffolds proved to be extremely cytocompatible and enhance cellular proliferation

and alkaline phosphatase secretion in MG-63 cell line, which proves its promising role to be used as biomaterial in bone tissue engineering (Venkatesan et al. 2014a, 2014c). Alginate–polymer composite (PLGA, PEG, and chitosan) (Choi et al. 2010; Reyes et al. 2014; Jeon et al. 2014; Li et al. 2016; Do et al. 2017), alginate–protein (collagen and gelatin) (Sotome et al. 2004; Eslaminejad et al. 2007; Xia et al. 2012; Lee et al. 2012a; Bendtsen and Wei 2015; Leena et al. 2017; Luo et al. 2018), alginate–bioglass (Chen et al. 2013, 2014; Rafienia et al. 2017), alginate–biosilica (Gabbai-Armelin et al. 2014; Müller et al. 2015; Zhang et al. 2017), alginate–bone morphogenetic protein-2, alginate-transforming growth factor-β1 (Reyes et al. 2014), IGF-1 and BMP-6 releasing chitosan/alginate/PLGA hybrid (Duruel et al. 2017) and RGD peptides (Mata et al. 2011; Bidarra et al. 2011) were extensively studied by several researchers. Hence, alginate-based composite materials show its potentiality for bone tissue engineering applications for the inherent qualities like controlled porosities, good mechanical strength, enhanced cell adhesion, cellular proliferation, increased biocompatibility, cellular mineralization, and differentiation of mesenchymal stem cells into osteogenic cells (Venkatesan et al. 2015).

9.1.6 Biosilica

Biosilica or biogenic silica is the form of glassy amorphous silica-SiO_2, containing water and small amounts of Al, Ca, Cl, Cu, Fe, K, Na, S, and Zn (Ehrlich et al. 2017), derived mainly from marine sponges, diatoms, radiolarians, and choanoflagellates (Schröder et al. 2008). Biosilica is proved to be magnificently important for bone formation as it has positive effect on osteoblast formation and abrogate osteoclast formation as well (Müller et al. 2009; Wiens et al. 2010; Wang et al. 2014b). In vitro studies showed that biosilica upregulates the gene expression of bone-forming cells and enhances mineralization and cell proliferation (Wiens et al. 2010; Granito et al. 2017). Several clinical and experimental studies with biosilica depicted its osteogenic potential and confirmed the ability of a promising biomaterial for drug delivery (Wang et al. 2012, 2013; Müller et al. 2015; Cicco et al. 2015; Özarslan and Yücel 2016; Tamburaci and Tihminlioglu 2018; Cruz et al. 2020).

9.1.7 Cuttlefish

The fishbone derived from cuttlefish (phylum Mollusca, class Cephalopoda) has similar chemical and structural properties like hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) (Manoli and Dalas 2000), which is considered to be most popular for preparation of scaffold for clinical use and bone tissue engineering till date (Dorozhkin and Epple 2002; Kim et al. 2014). Studies depicted that porous polycaprolactone (PCL) scaffold prepared using cuttlefish bone-derived HAp (CB-HAp) powder produces better osteogenesis than PCL alone (Kim et al. 2014). Hydroxyapatite derived from cuttlefish bone has unique properties such as better biocompatibility, increased cellular proliferation, and increased biomineralization from mesenchymal stem

cells and osteoblastic cells, and these properties can be further enhanced by incorporation of bioglass, polycaprolactone, and polyvinyl alcohol (Venkatesan et al. 2018). Further, compressive strength of cuttlefish bone-derived hydroxyapatite scaffold can be enhanced by polycaprolactone coating (Kim et al. 2014; Milovac et al. 2014). Ion (Sr^{2+}, Mg^{2+}, and/or Zn^{2+})-doped biphasic calcium phosphate (BCP) scaffolds prepared from cuttlefish bone (CB) with polymeric coating yields scaffold with better osteogenic properties and compressive strength (Neto et al. 2019). Hence, cuttlefish bone-derived hydroxyapatite is most appropriate scaffold biomaterial in bone tissue engineering (Rocha et al. 2005; Battistella et al. 2010, 2012).

9.1.8 Sponge

Marine sponges are the main organism sorted under the phylum Porifera, which enhances bone formation in a very promising way. Calcium carbonate ($CaCO_3$) constitutes main skeletal structure of sponge; moreover, a protein named spongin (contains collagen) and sometimes siliceous spicules are also present in its structure (Castro and Huber 2019). In vitro study with *Chondrosia reniformis* shows that collagen derived from sponge along with silica, when prepared as a form of hydrogel, helps in cellular growth and attachment in osteoblast-like cell line (Heinemann et al. 2007b). Several other components like pseudokeratin, neurokeratin, horny protein, and collagen like protein derived from sponges have various biological roles along with treatment of osteoarthritis. Another in vitro study with *Hymeniacidon sinapium* depicted that sponging enhances bone mineralization, increases collagen production, and enhances secretion of osteocalcin and alkaline phosphatase activity in MG-63 cell line. Study also concluded that spongin decreases the production of inflammatory mediators like TNF-α, IL-1β, and PGE_2, as a result acts as potent anti-inflammatory agent in orthopedic complications (Kim et al. 2009). Besides, molecules derived from sponges have many other activities like antiviral, antibacterial, antitumor, and osteogenic properties (Granito et al. 2017). Spongin (SPG) when incorporated into hydroxyapatite improves the graft properties and bone regeneration (Parisi et al. 2019; Fernandes et al. 2019) and serves as better graft substitute for orthopedic application. Study with cranial bone defect in rats showed SPG and biosilicate (BS) composite scaffold significantly enhance osteogenesis (Parisi et al. 2020). Hence, marine sponges are potential material for scaffold preparation due to its chemical properties (Jesionowski et al. 2018).

9.1.9 Echinodermata

Under phylum Echinodermata, sea stars (class Asteroidea) and sea urchins (class Echinoidea) are important organisms for bone regeneration as their ossicles are made from magnesium-rich calcite enhance osteoblast and bone marrow stromal cell adhesion to porous scaffold (Martina et al. 2005). This magnesium-rich calcite proved to be effective alternative for complicated ion substitution technique,

which enhances the osteogenic and structural properties of orthopedic implants (Kannan et al. 2007). Several studies showed that many organisms under this phylum also possess antibacterial property (Villasin and Pomory 2000; Haug et al. 2002) that may be beneficial for various orthopedic applications.

9.2 Desirable Properties of an Ideal Scaffolds as Drug Delivery Systems

The main objective of bone tissue engineering is to enhance the osteosynthesis and replacement of damaged or diseased bone with suitable biomaterials, cells, and/or bioactive molecules (Saltzman and Olbricht 2002). Though allografts are considered as ideal material for this purpose, limited availability and associated donor site infection, possibilities of disease transmission have limited its use for bone tissue engineering (Langer 2000). Hence, biodegradable scaffolds made with polymers have gained much importance as an alternative as they are able to deliver cells, genes, and/or drugs and thus enhance in-growth of osteogenic cells and overall modulate bone tissue regeneration (Freed et al. 1994; Hutmacher 2000). There are various types of polymeric scaffolds that possess three main properties that either alone or in combination evolves ideal scaffold material for bone tissue engineering: scaffolds a) which accommodates the cells in original physiological environment, b) cells capable of producing tissue, and c) growth factors and other biomolecules that guides the accommodated cells to differentiate into desired type (Garg et al. 2012).

To be an ideal material for drug delivery, the scaffolds should possess the characteristics such as:

(a) Drug should disperse throughout the scaffold in a uniform manner.
(b) Drug should elute from the scaffold in a desired rate.
(c) Drug should bind with the scaffold with a low affinity so that, during incorporation, it should be stable at physiological temperature.
(d) The physical, chemical, and biological structure of scaffold should be stable for long duration in vivo.
(e) Scaffold should have desired level of biocompatibility and toxicity level.
(f) Scaffold should be biodegradable and degraded metabolite should be nontoxic to tissues.
(g) Scaffold should have similar mechanical properties with the tissue of implantation.
(h) Scaffold should facilitate cell proliferation, adhesion, and migration.
(i) Scaffold should have recognizable macroscopic and microscopic structure, which facilitates cell or drug adhesion.
(j) Scaffold should be porous with adequate pore size and number.
(k) Scaffold should mimic the endogenous native extracellular matrix (ECM).
(l) It should be easily processable and moldable to desired shape and size.
(m) Scaffold should have higher loading capacity kinetics, so that drug elute in a continuous manner for longer period.

Targeted delivery of pharmaceutical agents has been foremost issue of interest of researchers and clinicians over conventional therapeutic approach as they have many advantages like specificity to local area, controlled dosage, and duration with less side effects. Numerous organisms of marine origin like shells, corals, chitosan, and sponges also provide ideal material in tissue engineering and drug delivery system owing to their unique chemical compositions and porous structure with interconnected pores.

9.3 Commonly Available Marine Materials Related to Bone Disorder Management

Though management of different bone disorder needs different types of treatment protocol, from the perspective of regenerative medicine, bone healing is established up on a triad: cellular part, scaffold that facilitates bone tissue growth, and incorporation of biomolecules or growth factors (Ringe et al. 2002). In this context, marine organisms have potential role in preparation of scaffold and promotion of osteogenesis as well through acting as a source of osteogenic bioactive (Table 9.1).

9.4 Marine Materials as Delivery Vehicle of Growth Factors/Peptides for Nonunion Fractures

Healing of fracture is considered as a unique physiological cascade, in which primary union is considered as ideal type, where soft callus gets structural and geometrical resemblance with normal bone after getting mineralized with time by the process of remodeling (Mosekilde and Bak 1993). But, study shows 5–10% of fracture complications occur in the form of delayed or nonunion (Tzioupis and Giannoudis 2007; Einhorn and Gerstenfeld 2015). There are certain controlling factors, which contribute to the development of fracture complications, e.g., senility, infection, deficiency of nutrients, excessive use of anti-inflammatory drugs, diabetes, smoking, and nicotine. Autologous bone grafts have been used since decade to treat nonunion, but it has certain limitations too. Present concept of treating this type of fracture is to provide components like mesenchymal stem cells and/or growth factors into osteoconductive scaffolds along with providing optimum mechanical and physiological environment. This concept is known as "diamond concept" (Fig. 9.2), and it provides optimum requisites for fracture healing (Giannoudis et al. 2007).

For this purpose, marine biomaterials have undergone through several in vitro and in vivo researches, which proved its uniqueness due to the nontoxic, biodegradable, and biocompatible characters. Marine polysaccharide is obtained from algae, prokaryotes, skeletons of crustaceans, and cartilaginous fish, though mainly used in food and cosmetic purpose, but also has enormous potential to be used as such bioactive material, which can deliver bioactive agents to the tissue (Laurienzo 2010). Marine polysaccharides have various chemical and biological properties like biocompatibility, biodegradability, and adhesive properties, and they can easily form

Table 9.1 Marine materials as osteogenic material

Compound	Bioactive/extract	Role	Reference
Fucoidan extract	Fucoidan	Osteoconduction	Changotade et al. (2008)
Fucoidan scaffold	Fucoidan	Osteoconduction Osteogenesis	Lu et al. (2019)
Hydroxyapatite–fucoidan (HApF) nanocomposite	Fucoidan	Enhanced bone mineralization twice than HAp alone	Jeong et al. (2013)
Chitosan–natural nano-hydroxyapatite–fucoidan nanocomposite scaffold	Fucoidan	Enhances bone mineralization	Lowe et al. (2016)
N,O-carboxymethyl chitosan/fucoidan conjugate nanocomposite scaffolds	Fucoidan	Enhanced cell proliferation, ALP activity, and mineralization of osteoblast	Lu et al. (2018)
Coral and adipose tissue-derived stem cells (ASCs) scaffold	Coral cells	Bone regeneration in cranial critical size defect in canine model	Liu et al. (2013)
Coral/hydroxyapatite implant with growth factor	Coral cells	Osteogenesis	Nandi et al. (2015)
Coral scaffold with autologous Mesenchymal stem Cells	Coral cells	Bone regeneration	Manassero et al. (2013)
Coral scaffold with BMSC, BMP2, and VEGF	Coral cells	Bone regeneration	Xiao et al. (2011)
Porous nanohydroxyapatite/coralline blocks coated with $rhVEGF_{165}$	Coral cells	Angiogenesis and bone regeneration	Du et al. (2015)
Tubular coral scaffolds with cell sheets	Coral cells	Osteogenesis	Gao et al. (2009)
Chinese soft coral *Lobophytum pauciflorum*	Lobophytone A-G	Anti-inflammatory	Yan et al. (2010)
Soft coral *Sinularia gyrosa*	Gyrosanols A-C	Anti-inflammatory	Cheng et al. (2010)

(continued)

Table 9.1 (continued)

Compound	Bioactive/extract	Role	Reference
Brown seaweed	Fucoxanthin	Hinders oxidative stress-related RANKL-mediated osteoclastogenesis	Kose et al. (2016)
Brown algae (*Sargassum siliquastrum*)	Sargachromanol G	Inhibits osteoclastogenesis and osteoporosis, anti-inflammatory	Yoon et al. (2012a, b, 2013)
Brown algae (*Sargassum thunbergii*)	Quinone derivatives	Anti-adipogenic and pro-osteoblastogenic	Kim et al. (2016)
Red algae (*Laurencia undulate*)	Floridoside	Osteogenic differentiation	Ryu et al. (2015)
Calcareous red algae (*Lithothamnion corallioides*)	Aquamin	Helps in mineralization of osteoblast and osteogenesis, and improves mechanical strength of collagen graft	Frestedt et al. (2009); O'Gorman et al. (2012); Widaa et al. (2014); Brennan et al. (2015)
Cyanobacteria (*Lyngbya* sp)	Biselyngbyaside	Inhibits osteoclastogenesis and induces apoptosis in mature osteoclasts	Yonezawa et al. (2012)
Dinoflagellate (*Symbiodinium* sp)	Symbioimine	Antiresorptive agent, prevent osteoporosis	Kita et al. (2004); Woo et al. (2008)
Microalgae	Peptide	Osteoblastic differentiation of MG-63 cells, anti-inflammatory	Qian et al. (2018); Carson and Clarke (2018); Suttisuwan et al. (2019)
Prokaryote	Polysaccharide	Bone and cartilage repair	Rederstorff et al. (2011)
Cyanobacterium	Largazole (depsipeptide)	In vivo and in vitro osteogenesis, rheumatoid arthritis inhibitor	Lee et al. 2011; Ahmed et al. (2013)
Sponge	Phorbaketal A	Osteoblastic differentiation of mesenchymal cells, anti-osteoporotic	Byun et al. (2012); Senthilkumar et al. (2014)

hydrogels (Costa et al. 2010; Ngo and Kim 2013). Several studies depicted that these biomaterials are able to load even very low drug dosage; as a result, side effects due to drug can also be minimized and they can also act as a marker, which delivers the signal to the vehicle to dispense drug at specific location (Allen and Cullis 2004; Brannon-Peppas and Blanchette 2004). It has proved its efficacy over viral vector in delivering gene also, through which health risk can be minimized (Thomas et al. 2003). Being biocompatible and biodegradable as a unique biomaterial, it has

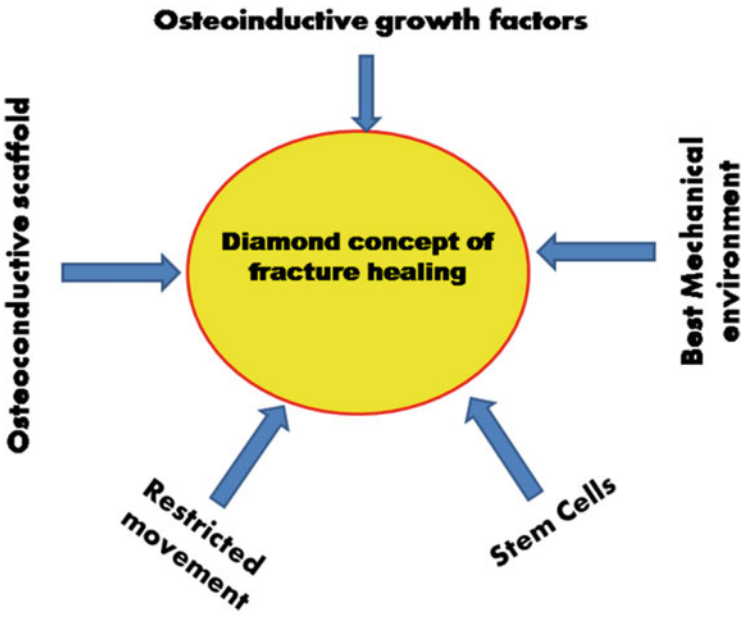

Fig. 9.2 Diamond concept of fracture healing

capability to stabilize gene and other therapeutic molecule by encapsulating, as well as promote sustained release of the agent to the host cells (Silva et al. 2012; Nitta and Numata 2013). Chondroitin sulfate, alginate, chitosan, hyaluronan, etc., fall under this group. Study with alginate/nanofiber-based hybrid growth factor delivery system with recombinant bone morphogenetic protein-2 (rhBMP-2) in critical size bone defect in rat depicted promising result in the treatment of bone injury and successful delivery of growth factor (Kolambkar et al. 2011). Similarly, marine sponge skeleton made scaffold is suitable for osteogenic factor delivery as well act as conductive and inductive framework for attachment of human stem cell (Green et al. 2003). Researches show glycosaminoglycan (GAG) is well capable of delivering different growth factors and cytokines in the target tissue as well as itself possess potentiality for repair of fibrocartilage (Hachim et al. 2019). Researches show that porous chondroitin-4-sulfate (CS)–chitosan sponge that release platelet-derived growth factor-BB (PDGF-BB) has potential role in bone regeneration (Park et al. 2000). Chitosan–gelatin scaffolds are capable of delivering basic fibroblast growth factor (bFGF) and bovine serum albumin (BSA) successfully (Azizian et al. 2018). On the other hand, chitosan-based vehicle is capable of delivering BMP-2 in vitro and in vivo (Venkatesan et al. 2017). Composite hydrogel with incorporation of poly (lactide-co-glycolide) (PLGA) and *2-N,6-O*-sulfated chitosan (26SCS) loaded with rhBMP-2 and angiogenic rhVEGF$_{165}$ serves as ideal material for growth factor delivery and improves angiogenesis (Cao et al. 2018). Another study depicted chitosan sponge with platelet-derived growth factor-BB (PDGF-BB) enhances

osteogenesis (Jeong Park et al. 2000) and chitosan/tricalcium phosphate (TCP) sponge carrier acts as potential carrier for growth factor delivery (Lee et al. 2000). Calcium phosphate cement scaffold made with tetracalcium phosphate [TTCP: $Ca_4(PO_4)_2O$], dicalcium phosphate (DCPA: $CaHPO_4$), chitosan, and mannitol pyrogen proves to be excellent in mechanical strength and capable of delivering osteogenic cells and osteoinductive growth factors (Xu et al. 2008). Chitosan-based scaffolds are capable to sequentially deliver BMP-2 and BMP-7 to the target tissue, hence are good candidate for growth factor delivery and osteosynthesis (Yilgor et al. 2009). Studies also depicted that chitosan–alginate (Ch-Al) composite can deliver growth factor in a controlled and sustained manner and help in cartilage regeneration (Reed and Wu 2017). Collagen/chitosan microgranules loaded with TGF-β1 show enhanced osteogenic capacity in the rabbit calvarial defects (Lee et al. 2006). From the abovementioned citations, it is evident that marine bioactives can effectively deliver osteogenic agents like growth factors, cells, and other biomolecules to the target tissue and can serve effectively for the purpose of treatment of different bone-related pathological conditions including nonunion fractures as a result escalate the quality of life of many patients.

9.5 Marine Materials as Delivery System of Drugs for Treatment of Osteoporosis

In the coming years, osteoporosis (OP) is going to be the major important diseases of concerns for the elderly patients (Johnell and Kanis 2006; Blume and Curtis 2011; Wright et al. 2014). In OS, the architecture of bone is lost along with loss of bone strength and altered macro geometry of bone. The conventional treatment for osteoporosis includes administration of antiresorptive agents like estrogen, calcitonin, bisphosphonates, denosumab, and strontium ranelate, anabolic agents like recombinant human parathyroid hormone, and different bioactive agents like odanacatib and cathepsin-K (Mauck and Clarke 2006; Brennan et al. 2009; Ruckle et al. 2009; Gesty-Palmer et al. 2009; Hannon et al. 2010; Glantschnig et al. 2011; Y Maximov et al. 2013; Canalis 2013; Iyer et al. 2014; Wang et al. 2014a; Ponnapakkam et al. 2014; Roskoski 2015; Appelman-Dijkstra and Papapoulos 2016), either involves oral administration of tablets or in the form of injections, which most of the times are associated with variable absorption, low bioavailability of the drugs at lower clinically effective dose, and difference in patient compliance, and therefore, many of occasions cannot change the prevailing pathological conditions of OP (Luhmann et al. 2012). Therefore, on-target supply of drugs with more efficient methods of therapeutic delivery is required.

Hydrothermally converted foraminifera exoskeletons with multistimulatory ion-doped marine scaffold have been evaluated as a local drug delivery system in OP (Chou et al. 2013). In this study, the scaffold was implanted intramuscularly in close proximity to the femur bone of mice, which were ovariectomized to create OP model, and the bones were evaluated and found that Zn-doped scaffold was proved to be better for OP bones. Zn is an essential component of cell metabolism and

possesses the ability to stimulate osteoblastic activity (Otsuka et al. 2008; Kannan et al. 2011; Luo et al. 2014). In rats, different types of Zn-injectable powder have been evaluated in OP (Kawamura et al. 2000; Sogo et al. 2002; Otsuka et al. 2004; Tokudome et al. 2011). Guo et al. (2015) reported better calcium absorption and enhanced femur bone mineral density in vivo in rats by core-shell MCP chelated calcium/calcium alginate nanoparticles produced from Synodontidae fish scales. The result is encouraging in terms of calcium supplementation in OP bones.

9.6 Marine Biomaterials as Delivery of Antibiotics in Treatment of Osteomyelitis

Osteomyelitis is the foremost challenge in orthopedic surgery because of multifactorial reasons of ineffectiveness with conventional antibiotic therapy (Prasanna and Venkatasubbu 2018). In general, osteomyelitis is managed by debridement, foreign body removal, and subsequent parenteral antibiotic administration (Schlossberg 1988). The overall cost of the treatment episodes is increased due to more hospital stay, cost of medicines, and additional surgical interventions besides adverse side effects of drugs. Alternatively, application of local drug delivery is being investigated by many workers (Gomes et al. 2013; Uskoković and Desai 2014a) and proved to be promising in terms of achievable effective dose of antibiotic at the infection site and without systemic toxicity. PMMA cement beads (Josefsson et al. 1990; Stabile and Jacobs 1990; Grime et al. 1990), collagen sponge (Trafny et al. 1995, 1996), biodegradable polymers from glycolide and lactide (Nie et al. 1995; Benoit et al. 1998), and calcium phosphate-based ceramics (Arita et al. 1995; Zafirau et al. 1996; Lemons 1996; Tampieri et al. 1997; Fanovich and Porto Lopez 1998; Ghosh et al. 2008; Nandi et al. 2008a, b, 2009) are the most commonly studied drug delivery systems in osteomyelitis. Ceramics and other materials incorporated to the thin films and bulk composites have been studied previously as biodegradable drug delivery systems with the limitations of unfitting to any bone defect size. Thus, marine resources such as marine shells, foraminifera, and corals have been the recent trend of research as an alternative drug delivery system.

Marine resources are also well explored as a new virgin area of research for drug delivery in osteomyelitis. Chitosan, a naturally derived polymer, may be easily extractable from crustacean shells. It has got antimicrobial, antitumor, anti-inflammatory, and immunity improving properties (Smelcerovic et al. 2008; Yin et al. 2009; Ing et al. 2012; Arancibia et al. 2013) may increase the permeability of many drugs due to its mucoadhesive characteristics (Kowapradit et al. 2010; Yeh et al. 2011). Nanoparticulate composites of hydroxyapatite (HAp) and a marine polymer, chitosan, were evaluated in osteomyelitis to overcome the burst release of small molecules from well-dispersed HAp nanoparticles (Uskoković and Desai 2014b). Different drug delivery systems of chitosan have previously also been studied (Wilson and Hull 2008; Palazzo et al. 2011; Tanase et al. 2012; Tripathi et al. 2012; Chen et al. 2012; Lee et al. 2012b). Wang et al. studied poly(vinyl alcohol) (PVA) and carboxymethyl chitosan (CMCS) drug delivery system loaded

with ornidazole (OD) both in vitro and in vivo and found to have effective antimicrobial efficacy for 5 days post-transplantation in rat subcutaneous tissue in a dose-dependent manner with CMCS (Wang et al. 2007).

9.7 Marine Biomaterials as Delivery Carriers of Drugs/Stem Cells for Management of Osteoarthritis

Chondroitin sulfate (CS), a natural glycosaminoglycan, is found in the cartilage and extracellular matrix. It has got beneficial effects in osteoarthritis. Cow trachea, pig ear and nasal septa, chicken keel, shark cartilage, and fish are the commonest source of CS for clinical and commercial trial (Nakano et al. 2000; Luo et al. 2002; Lignot et al. 2003; Nandini et al. 2005; Vázquez et al. 2013).

Oral administration of chondroitin sulfate with glucosamine helps to repair or slow-down the progress in OA by reducing cytokines and transcription factor concentration. Glucosamine increases cartilage-specific matrix components and thus reduces collagen degeneration. Chondroitin-S plus glucosamine is slow-acting drugs to reduce pain and provide functional stability of the joint to some extent. The clinical benefits of CS administration in patients with OA have been observed in various studies (Uebelhart et al. 2004; Prabhakar and Sasisekharan 2006; Clegg et al. 2006). Liposome-entrapped chondroitin in clinical studies has also been demonstrated (Trif et al. 2008).

9.8 Marine Materials as Local Delivery of Drugs for Treatment of Bone Cancers

Recent research on cancer therapy explored the vast potentiality of marine bioactive compounds (Reese et al. 1996; Pettit et al. 1998), especially mollusk as a vital source for developing chemotherapeutic agents against tumor cells. Mollusk provided some key anticancer compounds like dolastatin (Pettit et al. 1998), acharan sulfate, an innovative type of glycosaminoglycan from the giant African snail *Achatina fulica* (Lee et al. 2003), Kahalalide F (García-Rocha et al. 1996), and kulolide (Reese et al. 1996). There is a paramount necessity to explore an ideal marine-based compound for delivery system of anticancerous drug.

Bone, a complex tissue, may face defects by trauma, neoplastic growth, hereditary defects, accidents, osteoporosis, arthritis, etc. Different kinds of grafting like auto, allo, and xeno have been carried out to mitigate these problems, but all of these procedures have both advantages and limitations. Different marine biomaterials have been investigated out of which alginate is one of the most studied one. Alginate, a biopolymer found in seaweed, comprising of guluronic acid and mannuronic acid, is nontoxic, nonimmunogenic, and biocompatible. The most advantage of alginate is that it can be noninvasively introduced in any bone defect; it can easily fill irregularly shaped defects and can in a controlled manner deliver effectively the BMP and TGF-β (Krebs et al. 2010; Lópiz-Morales et al. 2010; Kolambkar et al.

2011). Alginate hydrogels have been explored as drug delivery system (DDS) for delivery of stem cells in bone tissue engineering (Barralet et al. 2005). Preferential adhesion of alginate to osteosarcoma cells has been investigated in combination of porous scaffold and found to be very effective (Lin and Yeh 2004; Turco et al. 2009). Zhao et al. (2012) demonstrated that alginate/$CaCO_3$/DNA/DOX nanoparticles show its potentiality in cancer treatments as alginate/$CaCO_3$/DNA/DOX nanoparticles could successfully mediate gene transfection and carry the drug to the target cells (Zhao et al. 2012). Alginate-incorporated calcium phosphate nanoparticles showed antitumor activity on human osteosarcoma in a dose- and time-dependent manner, and the drugs were found to release at a faster rate in relation to pH because of pH-dependent dissolution of CaP. These findings encouraged the researchers to think the drug-loaded CaP nanocomposites as newer controlled drug release vehicles for chemotherapy in cancers, especially in osteosarcoma (Son and Kim 2017).

Chitosan, a linear polysaccharide of glucosamine, can be obtained from chitin. Chitins are usually found in fungi, diatoms, nematodes, arthropods, shrimps, crabs, lobsters, krill, and squid (Teng et al. 2001; Cauchie 2002; Cira et al. 2002; Synowiecki and Al-Khateeb 2003; Rao and Stevens 2006; Hayes et al. 2008; Khanafari et al. 2008; Fai et al. 2011). Significant inhibitions of tumor growth have been demonstrated by chitosan (Carreño-Gómez and Duncan 1997). The antiproliferative efficacy of chitosan in breast cancer has been evaluated by intratumoral administration by Chen et al. (1997). Extensive research has been carried out to develop effective and safe chitosan-based drug delivery system (van der Lubben et al. 2001; Alonso and Sánchez 2003; Tiyaboonchai 2003; Hejazi and Amiji 2003; Kumari et al. 2010; Amidi et al. 2010; Bhattarai et al. 2010). Drug delivery in a sustained manner by chitosan-based particulate in different routes has also been investigated (Alonso and Sánchez 2003; Tiyaboonchai 2003; Hejazi and Amiji 2003; Kumari et al. 2010; Amidi et al. 2010; Bhattarai et al. 2010).

Novel pH-sensitive drug delivery system-based cockle shell-derived aragonite nanoparticles against osteosarcoma have been studied by Fu et al. (2017). Cockle shell (*Anadara granosa*), a sea species, may be considered as rich source of biogenic aragonite (Hoque et al. 2013). Aragonite is one of the polymorph forms of CaCO3. Biogenic calcium carbonate (CaCO3) being bioresorbable, osteoconductive, biocompatible, and slowly biodegradable may be served as an effective DDS in osteosarcoma model (Yu et al. 2010; Svenskaya et al. 2013; Zhao et al. 2015; Vergaro et al. 2015; Yang et al. 2016; He et al. 2016). Doxorubicin was loaded in Aragonite nanoparticles and was investigated in osteosarcoma with promising result.

9.9 Concluding Remarks and Future Direction

The sources of marine biomaterials are vast, and they can be converted to appropriate biocompatible scaffold for delivery of drugs/biomolecules both in hard and soft tissue engineering. Majority of materials of marine sources are biocompatible and structurally similar to human counterpart, and as such, they can successfully used in

biomedical application including drug delivery system. Some of the materials have inherent porous structure, which is highly suitable for drug delivery applications. A considerable stride has been made to search active ingredients in marine organisms before for biomedical applications. Yet, more effort should be put in harnessing active ingredients from marine species and biosynthesize them in laboratory in a large scale to fulfill the challenging demands in present days. This needs combined efforts by the researchers, scientists, teachers, and government organization how best we can utilize the abundant source of marine biomaterials in biomedical applications. However, we should not disturb the biodiversity of marine life.

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Expanding Role of Marine Natural Compounds in Immunomodulation: Challenges and Future Perspectives

10

Pavani Sanapala, Sudhakar Pola, N. Nageswara Rao Reddy, and Veera Bramchari Pallaval

Abstract

Marine flora is a source of novel compounds with biological activities. Over the years, thousands of marine organisms evolved in the habitat where each of them competes for a space of living, attachment, and survival. Many new compounds have been extracted from diverging marine organisms and were tested for therapeutic and pharmacological effects, where many are now available commercially. The screening of marine metabolites for antiviral, cancer, anti-inflammation, antimicrobial, and immunomodulation yielded a significant number of active crude and organic extract. The discovered compounds are taken into the marketplace with low cost-effective treatments. Gene modification at the laboratory level also helps in getting a potent compound capable of modulating the disease's biological pathway. New advanced techniques in culturing and extracting promising results should be of great concern and new trend for modern medicine. The secondary metabolites or the natural products derived from marine sources till now target bioactivities such as direct inhibition of enzymes in DNA synthesis and transcription. Few hinder metastasis by allocating as inhibitors, for example, matrix metalloprotease inhibitor. Few others have cytotoxic activity, while others obstruct the cell cycle phases or Rho-GTPase opposing tumor development. Here, the chapter gives detailed literature on the marine natural products

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discovered and illustrating their immunomodulation activities against various infectious, chronic, and nonchronic diseases and provides information about challenges and future perspectives.

Keywords

Immunomodulators · Marine flora · Antiviral agents · Marine drugs · Bioactive compounds · Anti-inflammatory activity · Cytotoxicity

10.1 Introduction

A result of modern biological variation is due to evolution happening from billions of years. Acclimatizing the habitat and optimizing the defense mechanism are obligatory to plants and animals because of the biological variations and processes. So far, 1.75 million species have been known as per the World Conservation Monitoring Centre reports (Groombridge and Jenkins 2000), yet researchers guesstimate the number to be fifty times more. Water bodies worldwide wharf a range of organisms with a majority of marine species dwelling in the vent communities and ocean fringe. These organisms have deficient substantial defense mechanisms due to controlled mobility, such as sessile organisms such as algae, sponges, corals competing for nutrients, anchoring, and survival. However, the organisms have a very complicated chemical defense mechanism based on the production of toxic derivatives such as sugar metabolites, alkaloids, peptides, steroids, and terpenoids. Over several thousand years, humankind has known the therapeutic potential of marine organisms and the active compounds able of effective biological activities. However, the interest in investigating and exploring marine-derived compounds and natural products has started from half a century ago. From the time, marine species, namely bacteria, sponges, algae, fungi, ascidians, corals, and many more, have been the understudy for the natural product content (Bhadury et al. 2006; Prudhomme et al. 2008).

Natural products are generally low molecular weight compounds referred to as secondary metabolites. Secondary metabolites are uncommon in comparison with primary metabolites. They are resultant of primary metabolisms such as amino acids, acetic acid, sugars, and biological alterations. Secondary metabolites from marine organisms have novel structures that include phenols, terpenoids, polyketides, steroids, alkaloids, proteins, few carbohydrates, and lipids with prominent pharmacological and biological activity. Based on biological function, secondary metabolites are further categorized as hormones, pheromones, antibiotics, oxylipins, toxins, phytoalexins, and so forth. The exploration of natural products is of ecological significance. However, few isolated marine compounds are known but differ from terrestrial due to the alerting variation in their secondary metabolism. Hence, isolating and synthesizing the marine-derived compounds are of great challenge to the researchers as the study joins up various biological techniques of almost all sciences. An imperative role played by natural products in recent years is the

establishment of new drugs. Drugs are medically suggested curative purposed for enhancing disease conditions, and reinstating good health and vitality to humans. Ocean derivatives and marine medicine are interrelated to restore health. It covers a diversity of practices with marine species to uphold health by curing and preventing medicine. Therefore, marine medicine is defined as the science of curing, reinstating, and maintaining health by treatment and prevention relative to the ocean derivatives. In recent days, comprehensive investigations of marine natural products lead to the discovery of novel compounds that influence the cellular metabolism and cell cycle (Molinski et al. 2009).

10.2 Immunomodulators

The term immunomodulator is a natural or synthetic substance that can amend or suppress, and stimulate the immune system. Immunomodulators are classified into immunoadjuvants (known to boost vaccine efficiency), immunostimulants (innately nonspecific, they foresee to fight against infections within the body), and immunosuppressants (heterogeneous drugs given in combination procedures to treat organ transplant rejection and autoimmune disease). Phytoelements such as didemnin, aplidine and trabectedin, polysaccharides, glycans, cyclic di- and tripeptides, carrageenan, sodium alginate, and many more constituents, the marine immunomodulators, act as natural products and have potential pharmacological activity (Costantino et al. 2009; Rathod et al. 2010; Chandraraj et al. 2010; Akerkar et al. 2009; Yim et al. 2005).

10.3 Significance of Marine Organisms

Over 50 years and so, the potential activity of marine compounds in cosmetic industries, nutritional supplements, agro- and fine chemicals, and treating various diseases is well-known (Tziveleka et al. 2003). However, since the past 30 years, the use of marine compounds in immunomodulation is quite promising. The novel compounds isolated from the marine species have unique characteristics such as antiviral, antibacterial, antimicrobial, antioxidant, anti-inflammatory, antitumor, analgesic, allergy, and immunomodulation. Besides these features, they also play a significant role in drug discovery and design for various human diseases, especially cancer therapy. Many compounds are under clinical and advanced preclinical trials (Newman and Cragg 2004). Marine species, both micro- and macroorganisms, are known to act as immunomodulators. Natural compounds from bacteria, particularly actinobacteria, cyanobacteria, and myxobacteria, algae, fungi, sponges, Chordata, Porifera, Plantae and Planktons, Arthropoda, Mollusks, Diatoms, and Bryozoans, act as modulators in stimulating the immune system. This is all because of the rich source of novel and bioactive marine organisms wherein the rate of compounds has been increasing year after year. Table 10.1 briefs the mode of action of a few natural compounds derived from marine species.

Table 10.1 Marine natural compounds and their mode of action as immunomodulators

Source	Chemical compound	Immunomodulatory activity	Reference
Bacteria			
Actinoalloteichus	Neomaclafungins A–I	Membered macrolides of the oligomycin significant modulation activity against *T. mentagrophytes*	Sato et al. (2012)
Aplidium albicans	Cyclic depsipeptide	Acts as anti-multiple myeloma in xenograft plasmacytoma murine model	Mitsiades et al. (2008)
Bacillus licheniformis	Glycolipopeptides ieodoglucomide A 10 and B 11; ieodoglucomide B; exopolysaccharide 1-T14 (EPS1-T14)	Have mild antimicrobial activity; ieodoglucomide B shows cytotoxic effect toward lung and stomach cancer; EPS1-T14 acts as a stimulator for TH1 cell-mediated immunity	Tareq et al. (2012)
Bacillus mojavensis	Iturinic lipopeptide Mojavensin A 12	Antifungal activity	Ma et al. (2012)
Bacillus subtilis	Amicoumacin and bacilosarcin analogs	Show cytotoxicity to HeLa cell and have antibacterial nature	Itoh et al. (1982)
Bryozoa neritina	Bryostatin polyketide	Antitumor activity	Kalechman et al. (1992), Philip et al. (1993)
Erythrobacter	Erythronic acids	Self-effacing activity for non-small-cell lung cancer cell	Hu et al. (2012)
Micrococcus luteus	Micrococcus antibodies	Immunosuppressive activity	Grooten et al. (1983)
Nonomuraea longicatena	Lestaurtinib (alkaloid)	Inhibits protein kinase and calmodulin	Ning et al. (2018)
Sorangium cellulosum	Exopolysaccharide	Hypoglycemia activity	Ding et al. (2004)
Salinispora tropica	Omuralide	A proteasome inhibitor, a potent inhibitor of human multiple myeloma	Prudhomme et al. (2008)
Salinosporamide	*Salinosporamide* A	Proteasome inhibitor works on various tumors, enhances apoptosis, and suppresses osteoclastogenesis through the NF-κB pathway	Ahn et al. (2007)

(continued)

Table 10.1 (continued)

Source	Chemical compound	Immunomodulatory activity	Reference
Tridemnum solidum	Didemnin B (depsipeptides)	Immunosuppressive and anti-inflammatory activity	Kijjoa and Sawangwong (2004), Ankisetty et al. (2013)
Sponges			
Clathria lissosclera	Clathriols (polyoxygenated steroids)	Hinders superoxide production	Keyzers et al. (2003)
Dendrilla nigra	Lipopolysaccharides	Secondary metabolites such as *D. nigra* have a potent activity for an antibacterial effect	Selvin and Lipton (2004)
Discodermia	Lactone compounds	Discodermia species showed immunosuppressive and cytotoxic activity	Longley et al. (1998)
Gelliodes fibrosa	Terpenoids, lipids, and steroids	Are the main compounds of *G. fibrosa* that acts as an immunostimulatory in combination with ethyl acetate	Chandraraj et al. (2010)
Haliclona sp.	Halipeptins	In vitro and in vivo anti-inflammatory activity	Randazzo et al. (2001)
Hyrrios	Puupehedione, dipuupehedione, bispuupehenone	The three major compounds isolated from *Hyrrios* have antiangiogenic, antitumoral, antioxidant, antimicrobial, immunomodulatory, and antiatherosclerotic effects	Martínez-Poveda et al. (2017)
Ircinia variabilis	Fasciculatin	Cytotoxicity activity	Rifai et al. (2005)
Lobophytum crassum	Lobocrassin B	Hampers lipopolysaccharide	Lin et al. (2013)
Petrosia sp	Petrocortyne A, contignasterol	Inhibits macrophages, histamines	Kim et al. (1999) Takei et al. (1994), Hong et al. (2003)
Reniera species	Sulfated polysaccharides (carbohydrates, uronic acids, sulfates)	Immunomodulation activity	Ahmad et al. (2019)

(continued)

Table 10.1 (continued)

Source	Chemical compound	Immunomodulatory activity	Reference
Tedania anhelans	Terpenoids, lipids, and steroids	Unlike *G. fibrosa*, *Tedania anhelans* has immunostimulatory property besides the chemotactic, phagocytic, and intracellular carnage in human neutrophils	Chandraraj et al. (2010)
Tedania ignis	Tedanol (diterpenoid)	Anti-inflammatory activity	Costantino et al. (2009), Castrillo et al. (2001)
Theonella swinhoei	(Cyclic peptides) perthamides C and D, barangamides B, C, and D, and theonellapeptolide	Anti-inflammatory activity exhibits a slight immunosuppressive activity	Festa et al. (2009), Festa et al. (2012)
Algae			
Chondrus ocellatus	Lambda-carrageenan	Antitumor activity, the proliferation of lymphocyte, NK cell activity	Zhou et al. (2004)
Crypthecodinium cohnii	Exopolysaccharide	Regulates the expression of modulators in the signaling pathway	Ma et al. (2017)
Eisenia arborea	Phlorotannin	Hinders immunoglobulin E; exhibits antidegranulation effects	Sugiura et al. (2008)
Eisenia bicyclis	Phlorotannins Dieckol, Eckol	Inhibits NO and lipopolysaccharide and induces ROS reaction	Shibata et al. (2003), Jung et al. (2013)
Endarachne binghamiae	Polysaccharides	Stimulates proliferation and enhances the production of TNF-alpha in macrophages	Huang and Lee (2005)
Fucus distichus	Phlorotannin subderivative	Reduces the expression of cyclooxygenase-2, interleukin-10, TNF-α, and monocyte chemoattractive protein-1	Kellogg et al. (2015)
Gracilaria verrucosa	Enone fatty acids	Hampers the production of TNF-alpha, nitric oxide, interleukin-6 biomarkers	Lee et al. (2009)
Gyrodinium impudicum	Sulfated polysaccharides	*G. impudicum* showed immunostimulatory effects and enhanced the tumoricidal activities of	Yim et al. (2004), Lee et al. (2008)

(continued)

Table 10.1 (continued)

Source	Chemical compound	Immunomodulatory activity	Reference
		macrophages and natural killer cells in vivo, sulfated polysaccharides of G. *impudicum* have antiviral property	
Hijikia fusiforme	Polysaccharides	Potential macrophage-stimulating effects enhanced pro-inflammatory activity	Jeong et al. (2015)
Laminaria japonica	Oligo- and polysaccharides	Apoptotic activity	Kim et al. (2006)
Meristotheca papulosa	Polysaccharides	The proliferation of human lymphocyte	Shan et al. (1999)
Monostroma nitidum	Sulfated polysaccharides	Stimulate macrophage cell line, inducing substantial nitric oxide production, suggesting as a strong immunomodulators	Karnjanapratum and You (2011)
Sargassum thunbergii	Fucoidan	Phagocytosis and chemiluminescence activity	Yende et al. (2014)
Spirulina fusiformis	β-Carotene, polysaccharide	Immunosuppression effect	Rasool and Sabina (2009)
Ulva fasciata	Lipopolysaccharide	The green algae *Ulva fasciata* in the diet significantly has anti-inflammatory and apoptotic activity against colon cancer	Anis et al. (2018), Ryu et al. (2013)
Fungi			
Neocosmospora vasinfecta	Cyclosporine	Inhibition of calcineurin (a calcium-dependent serine–threonine phosphatase)	Nakajima et al. (1988)
Penicillium sp.	Brevicompanine E	Lowers the production of pro-inflammatory cytokines	Yang et al. (2009)
Aspergillus insulicola	Azonazine (dipeptide)	Hinders the production of NF-κβ luciferase and nitrite	Wu et al. (2010)
Aspergillus sp. strain SF-5921	Aurantiamide acetate	Shows NF-κβ, c-Jun NH2-terminal kinase, p38 inhibition in BV2 microglia cells	Yoon et al. (2014)

(continued)

Table 10.1 (continued)

Source	Chemical compound	Immunomodulatory activity	Reference
Ascomycota sp. strain CYSK-4	Isocoumarins	Hinders the production of nitric oxide	Chen et al. (2018)
Xylaria sp. strain 2508	Xyloketal	Shows in vivo and in vitro neuroprotective activity on neonatal hypoxic–ischemic brain injury	Xiao et al. (2013)
Eurotium amstelodami	Questinol (anthraquinone)	Inhibits nitric oxide and prostaglandin E2 production	Yang et al. (2014)
Chaetomium globosum	Chaetoglobosin Fex	Immunosuppressive activity	Dou et al. (2011)
Ecklonia stolonifera	Phlorofucofuroeckol (phlorotannin)	Inhibits nitric oxide and prostaglandin E2 production by the restraining inducible nitric oxide synthase and cyclooxygenase-2 protein expression	Kim et al. (2009)
Macroorganisms			
Acorus calamus	Rhizome extract	Hinders cell proliferation	Mehrotra et al. (2003)
Bryozoans	Convolutamydine A	Hinders the production of interleukin-6, nitric oxide, inducible nitric oxide synthase (iNOS), cyclooxygenase 2, prostaglandin-2, and tumor necrosis factor-α	Fernandes et al. (2014)
Bugula neritina	Bryostatin 1	Combination with protein kinase C observed to have anticancer and immunostimulating activities	Iwu and Wootton (2002)
Carijoa sp.	Steroid glycoside carijoside	Inhibits elastase and superoxide	Liu et al. (2010)
Crenomytilus grayanus	Mytilan (bioglycan)	Isolated from *C. grayanus* mussel has high immunomodulating activity	Ovodova et al. (1992)

(continued)

Table 10.1 (continued)

Source	Chemical compound	Immunomodulatory activity	Reference
Ecteinascidia turbinata	Trabectedin	The antitumor drug decreases monocyte counts and differentiation of macrophages (ex vivo) in soft tissue sarcoma, chronic lymphocytic leukemia	Banerjee et al. (2019), Nakamura et al. (2016)
Euchelus asper	Methanol extract	Anti-inflammatory, antiproliferative, and *in ovo* antiangiogenic activity of methanol extract of *E. asper*	Agarwal et al. (2017)
Hemifusus pugilinus	—	Immunosuppressant activity	Ponkshe and Indap (2002)
Hyriopsis cumingiilea	Polysaccharides	Enhances immune response, especially adaptive immunity	Bai et al. (2009)
Litopenaeus vannamei	Polysaccharides	Superoxide dismutase performs as an immunomodulator and is used as a marker for immune response	Chiu et al. (2007)
Marthasterias glacialis	Ergosta-7,22-dien-3-ol	Anti-inflammatory	Pereira et al. (2014)
Mastigias papua	Polyketide sulfate	Inhibits inducible vascular cell adhesion molecule-1	Hanif et al. (2010)
Mytilus coruscus	Glucan	Immunosuppression	
Nematopalaemon tenuipes	Petroleum ether and ethyl acetate constituents	Stimulation and suppression activity	Akerkar et al. (2009)
Oily fishes	n-3 polyunsaturated fatty acids	Reduces the spread of human T cell, decreases the progression and onset of arthritis and other disorders such as swelling, knee pains	Miles and Calder (2012)
Rastrelliger kanagurta	Protein hydrolysate extract	Antioxidant function from the backbone of *R. Kanagurta*	Sheriff et al. (2014)
Sarcophyton sp.	Glycolipid and sarcoehrenosides	Hinders iNOS expression in macrophages	229,231
Seleronephthya gracillimum	Sclerosteroid	Hampers the expression of iNOS and cyclooxygenase 2 in macrophages	Fang et al. (2013)

(continued)

Table 10.1 (continued)

Source	Chemical compound	Immunomodulatory activity	Reference
Stichodactyla helianthus	Peptide	Regulates the function of effector memory T cells and class-switched memory B cells	Chi et al. (2012)
Tridemnum solidum	Didemnins A, B, and C, cyclic depsipeptides	Have antineoplastic activity, as well as the antiviral and immunosuppressive activities	Rinehart et al. (1988)

10.4 Marine Antiviral and Anticancer Compounds

Compounds and their metabolites isolated from marine sediments with varied biological activities ranging from antiviral to anticancer have been identified. Marine bacteria, algae, sponges, fungi, and macroorganisms are the primary source of antiviral compounds. The novel and unique marine antiviral agents (MAVAs) from the marine natural resources have multipotential uses with vast applications. The applications include (a) managing contamination and disease transmission of the enteropathogenic virus through sewage-contaminated waters; (b) chemotherapy for human viral diseases by increasing the yield and producing cost-effective drugs from MAVAs; and (c) finally, ruling out the diseases in marine animals by seeding MAVAs in the marine environment.

10.4.1 Marine Bacteria as a Source for Antiviral Agents

Bacterial-derived MAVAs are few with exopolysaccharide (EPS) marine antiviral agents as the main chemical constituents. Exopolysaccharides are the main component produced by marine bacteria, with unique features such as survival at unfavorable environment conditions categorized by extreme temperature and pressure, elevated levels of sulfides and metal concentration, attachment to the solid surface, and growth plan (Vincent et al. 1994). The mechanism of action of EPS on viruses is the hindering adsorption and entry of viruses into the host cell. The sulfated EPC does inhibit reverse transcription by restraining the reverse transcriptase enzyme. *Bacillus licheniformis* and *Geobacillus thermodenitrificans* isolated from shallow marine hot springs produce EPS1 and EPS 2, respectively. Both the exopolysaccharides inhibit HSV-2 replication in peripheral blood mononuclear cells by upregulating the pro-inflammatory cytokines and T-helper 1 cells (Arena et al. 2006, 2009). Bacteria inhabitant of deep-sea vents, namely *Vibrio diabolicus*, *Alteromonas macleodii*, *Alteromonas infernus*, and *Pseudoalteromonas* reference strain HYD 721, generates EPS alongside with macrolactin A compound, and the

Table 10.2 Antiviral compounds derived from marine sponges

Source	Mode of action	Reference
Petrosia sp.	Polyacetylenetriol inhibits RNA and DNA-dependent DNA polymerase activities of the retroviral reverse transcriptase of HIV; however, due to insufficient specificity, the compound is considered for the anti-AIDS drug rather than an anti-HIV agent	Loya et al. (2002)
Monanchora sp.	Crambescidin 826, a polycyclic guanidine alkaloid, acts as an in vitro fusion inhibitor on *the* HIV-1 envelope	Chang et al. (2003)
Petrosia similes	In vitro inhibition of HIV-1 replication, development of giant cell, and recombinant reverse transcriptase enzyme by a two bisquinolizidine alkaloids and petrosian	Goud et al. (2003)
Neamphius huxleyi	Neamphamide A, a novel depsiundecapeptide, hinders the cytopathic effect of HIV-1 infection	Oku et al. (2004)
Madagascan Lendenfeldia	C22 furanoterpene named as dehydrofurodendin is active against HIV-1 reverse transcriptase enzyme allied with RNA and DNA directed DNA polymerase	Chill et al. (2004)

latter show activity against HIV replication by protecting the human T lymphoblast cells. Macrolactin A reported having antiviral properties against HSV (Gustafson et al. 1989a; Gustafson et al. 1989b).

10.4.2 Antiviral Compounds Derived from Marine Sponges

Synthetic derivatives of arabinose nucleoside such as acyclovir, Ara-A (vidarabine), Ara-C (cytarabine), and azidothymidine (zidovudine) were isolated from *Tethya cripta,* a marine sponge. These metabolites show antiviral property against *herpes simplex virus* (HSV) (Elion et al. 1977; Privatdegarilhe and De Rudder 1964; Horwitz et al. 1964). New compound hamigeran B isolated from *Hamigera tarangaensis* showed activity against herpes and poliovirus (Wellington et al. 2000). Two metabolites weinbersterols A and B from *Petrosia weinberg* showed in vitro inhibition against leukemia and coronavirus in mice and feline leukemia (Sun et al. 1991). Researchers isolated three novel compounds in the search for inhibiting the infection of influenza virus in humans: calyceramides A, B, and C from *Discodermia calyx*, a marine sponge (Nakao et al. 2001).

The majority of antiviral agents derived from marine sponges show activity against HIV. One such species is *Dysidea avara,* a Mediterranean soft sponge that blocks the synthesis of UAG suppressor glutamine tRNA during HIV infection with sponge metabolites, avarol, and its derivatives (Müller et al. 1987; Boyd et al. 1996). Clathsterol is another novel metabolite isolated from *Clathria sp* (called red sea sponge) that revealed its active nature inhibiting HIV-1 reverse transcriptase (Rudi et al. 2001). Another novel HIV inhibitor, Microspinosamide, a cyclic depsipeptide, is synthesized from *Sidonops microspinosa* (Rashid et al. 2001). Table 10.2 details a few more MAVAs isolated, synthesized, and characterized by marine sponges; they play a critical role in inhibiting HIV.

10.4.3 Antiviral Compounds Derived from Marine Algae

Marine algae as a source for antiviral agents showed promising results. Metabolites extracted from algae have both in vivo inhibition activity and in vitro inhibition activity for many viruses. Major of the algal MAVAs are polysaccharide-derived. Sulfated polysaccharides A1 and A2 produced extracellular from marine algae *Cochlodinium polykrikoides* inhibiting influenza A and B viruses, and respiratory syncytial virus. Also, A1 and A2 are active against HSV-1 and parainfluenza type 2 virus (Hasui et al. 1995). Another sulfated polysaccharide compound named calcium spirulan isolated from the marine blue-green alga, *Arthrospira platensis,* has significant activity against HIV by reducing viral replication (Hayashi et al. 1996). A new polysaccharide compound, galactan sulfate, from *Agardhiella tenera*, a red seaweed, inhibits both type 1 HIV and type 2 HIV (Witvrouw 1994). The compound is also active against herpesviruses, arenaviruses, and togaviruses. Fucoidan isolated from brown algae *Fucus vesiculosus* (Béress et al. 1993) is shown to have activity against type 1 and type 2 of HSV, and reverse transcriptase of HIV 1 and vesicular stomatitis virus. Anti-HIV viral compounds cyanovirin-N (Boyd et al. 1996), diterpenes, and griffithsin (Mori et al. 2005) that inhibit viral replication of HIV have shown promising outcomes. However, diterpenes show activity on RNA-dependent DNA polymerase of the viral reverse transcriptase (Pereira et al. 2004). A novel compound naviculan, isolated from a diatom *Navicula directa,* shows activity against HSV type 1 and type 2 by hindering the main biological mechanism such as replication, adherence, and replication (Lee et al. 2006).

10.4.4 Fungi-Derived MAVAs

Marine fungal compounds such as equisetin and phomastein extracted from *Fusarium* and *Phoma* species, respectively, reported significant HIV (Singh et al. 1998). Another new compound isolated from *Fusarium* sp. is sansalvamide A that has inhibition activity toward *Molluscum contagiosum*, a poxvirus. Sansalvamide inhibits the virus by reducing topoisomerase activity that catalyzes DNA binding and relaxation and complex covalent interaction (Hwang et al. 1999). A derivative of halovir A to E compounds extracted from *Scytalidium* species showed significant antiviral activity against HSV type 1 and type 2 (Rowley et al. 2003). A novel terpenoid stachyflin isolated from *Stachybotrys* reported antiviral property against influenza A virus by inhibiting fusion among the viral envelope and host cell membrane (Minagawa et al. 2002).

10.4.5 Antiviral Compounds from Marine Macroorganisms

Besides microorganisms, compounds isolated from marine macroorganisms do have antiviral properties, many of which have anti-HIV activity, namely

cyclodidemniserinol trisulfate, didemnaketals A and B isolated from species of *Didemnum,* an Ascidian, the duo act against HIV by inhibiting HIV 1 integrase and protease, respectively (Mitchell et al. 2000; Potts et al. 1991). Compounds lamellarin isolated from *Lamellaria* and didemnid ascidians, polycitone A from *Polyctor* sp., polyphemusins I and II and tachyplesins I-III isolated from horseshoe crabs *Limulus polyphemus* and *Tachypleus testudinum,* respectively, and thalassiolins A-C isolated from seagrass, *Thalassia testudinum,*inhibit HIV hindering various activities (Reddy et al. 1999; Morimoto et al. 1991; Miyata et al. 1989; Rowley et al. 2002). Marine antiviral compounds inhibiting HSV are didemnins and eudistomin isolated from tunicates *Tridemnum solidum* and *Eudistoma olivaceum,* respectively (Hudson et al. 1988). Didemnins is potent against yellow fever, rift valley fever, and encephalomyelitis (Rinehart et al. 1988).

10.5 Marine Natural Compounds in Cancer Therapy

Chemotherapy is a treatment that kills cells with the aid of drugs especially that increase rapidly. Chemotherapy is often used to treat cancer by inhibiting cell division, growth, and expansion. Various drugs have been in use for cancer chemotherapy originated from nature. The significance of natural compounds as drugs is structural modification, and production of semi-synthetic and synthetic derivatives that yield effective drugs. The novel lead compound is in great demand as they lessen the side effects and enhances the bioactivity. Techniques such as high-throughput screening, computational chemistry, and genetic alteration have led to a new possibility for the invention and design of new anticancer drugs (Gordaliza 2007).

Over 10 years, the use of marine-derived natural compounds in trials against cancer has increased. For a natural product to aspirant as a drug, the compound should have the following criteria: (a) maintain sufficient industrial supply, (b) the compound shows moderate toxicity, (c) should have a risk to extend formulation under clinical trials, (d) analytical techniques to detect pharmacokinetic parameters, and (e) compound must be capable of metabolizing with maximum efficacy and restricting undesirable effects (Jimeno et al. 2004). Studies conducted in early years reported that marine microalgae, heterotrophic bacteria, and cyanobacteria existing within symbiotic associations with marine macroorganisms such as invertebrates are the top resources of biologically active compounds in cancer therapy (König et al. 2006). The mechanism of marine natural anticancer drugs is active targeting cellular and molecular activities. Usually, the compounds effectively hinder DNA polymerase or histone deacetylases for DNA synthesis and transcription, respectively (Bruserud et al. 2007). Other systems include inhibition of tumor metastasis and progression by matrix metalloprotease inhibitors and Rho-GTPase activity correspondingly, induction of apoptosis, and hindering NF-κβ activity (Welsh 2004). Anticancer compounds as drugs are mainly isolated from mollusk, sponges, and cyanobacterium. Few anticancer compounds derived from the marine source are listed below and the structures are detailed in (Fig. 10.1).

10.5.1 Acetylenic Lipids as an Anticancer Agent

Acetylenic lipids and their derivatives are products extracted from plants, fungi, or marine invertebrates. The compounds have varied functions in antimicrobial and antitumor therapies (75,76). Of all the marine species, sponges are more effective antitumor agents with cytotoxicity effects. Acetylenic alcohols produced from *Cribrochalina vasculum,* a brown bowl marine sponge, showed cytotoxic activity and reported antitumor activity against non-small-cell lung line. Derivatives of acetylenic lipids do have immunosuppressive activity (Dembitsky 2006).

Lembehyne A, a by-product of *Haliclona* species habitat of Indonesia marine, is a new long-chained polyacetylene compound that prevents the G1 phase of the cell cycle of neuroblastoma cells (Aoki et al. 2001). Few carotenoid metabolites are isolated from marine tunicates and mollusks, namely astaxanthin, carotene, cynthiaxanthin, and halocynthiaxanthin antimutagenic and anticancer effect against human cancer cell lines (Krinsky 1993).

A C24 acetylenic lipid, callysponginol sulfate A, produced from *Callyspongia truncate* affective toward membrane type 1 matrix metalloprotease inhibitor, results in the deterrence of metastasis (Fujita et al. 2002).

10.5.2 Aplyronine A

Aplyronine A (ApA) is an effective in vivo antitumor activity against murine leukemia, Ehrlich cancer, lung carcinoma, colon, and melanoma. The compound is isolated from *Aplysia kurodai,* a sea hare. The compound ApA has actin-depolymerizing activity when bound to tubulin in combination with actin putting off mitosis and spindle formation (Kita et al. 2017).

10.5.3 Bryostatin

Bryostatin isolated from a brown bryozoan, *Bugula neritina,* is a complex polyketide. Bryostatin series are effective activators of protein kinase C (PKC) with the anticancer property. The compound has passed the phase I clinical trial and is subjected to phase II human trials. The component is shown to be effective against renal carcinoma, melanomas, and lymphomas (Newman and Cragg 2007). Studies also reported the chemotherapeutic activity of bryostatin, but they can enhance cognitive function (Sun and Alkon 2006). It has a prominent role as an agonist to strengthen PKC and reduce Aβ levels and hindering tau protein as a therapeutic for Alzheimer's disease (Lucke-Wold et al. 2015).

10.5.4 Cytosine Arabinoside

Cytosine arabinoside (Ara-C), a pyrimidine analog, contains cytotoxic, antiviral, and immunosuppressive activity. Usually, nucleoside analogs in anticancer therapy comprise analogs of purine and pyrimidine nucleosides and nucleotides. These antimetabolites hinder the processes in the synthesis of nucleic acids, for instance, DNA replication. The cytotoxic effect exerted includes the intrusion of enzymes in nucleic acid synthesis. As the nucleoside analogs are hydrophobic, they require transporters to cross the cell membrane and pierce them. Ara-C is isolated from the Caribbean sponge *Cryptothethya crypta*is, a deoxycytidine compound. The compound is more effective against acute myeloid leukemia when injected intravenously (Newman and Cragg 2004).

10.5.5 Discodermolide

Discodermolide, a polyhydroxy lactone, is produced from the Caribbean sponge *Discodermia dissoluta*. Harbor Branch group first invented the compound in the year 1990. The compound has promising results of novel immunosuppressant activity along with cytotoxicity (Vaishampayan et al. 2000). The compound also hinders microtubules more effective compared to taxol. Discodermolide shows antitumor activity against breast, ovarian, colon, and multidrug-resistant cancers (Cuevas et al. 2000).

10.5.6 Dolastatin 10

Dolastatin 10 (DOLA-10) isolated from the mollusk *Dolabella auricularia,* a pentapeptide, was screened for anticancer property in 1972. The compound consists of distinctive amino acids such as dolaphenine, N,N-dimethylvaline, dolaisoleucine, and dolaproine. Derivatives of DOLA-10 were analyzed, but DOLA-10 showed high potent as an inhibitor for cell proliferation (Aherne et al. 1996; Madden et al. 2000). The compound is effective against tumor cell lines, namely melanoma, ovarian, and sarcoma cancer cells. DOLA-10 upregulates tumor suppressor protein (p53), the most common altered protein in human cancers (Madden et al. 2000). It aims to transactivation mechanisms by inducing cell cycle arrest, DNA repair, and apoptosis (Maki et al. 1995).

10.5.7 Ecteinascidin (ET)-743

ET-743 known as the first derived anticancer drug is a marine compound extracted from tunicate *Ecteinascidia turbinate*. An analog of ET-743 named ET-729 showed identical function and effectiveness with variation at the N terminal having a dimethyl residue. However, ET-743 is preferred more for research rather than

ET-729 as the second showed less availability. A synergistic effect is reported in studies with combinations of other resources, namely doxorubicin, irinotecan, and paclitaxel. The mode of mechanism showing antitumor activity is its binding to the DNA minor groove. Additionally, ET-743 inhibits transcription-coupled excision repair and provokes cell death (Takebayashi et al. 2001; Rinehart et al. 1990).

10.5.8 Hemocyanin

Keyhole limpet hemocyanin (KLH), a copper protein, is produced from the hemolymph of a marine mollusk *Megathura crenulata*. Hemocyanin was reported to have an immunostimulatory property in humans. The compound stimulated both humoral and cell-mediated immune responses. Oligomeric isoforms of KLH, i.e., KLH1 and KLH2, are revealed in the 1990s. Clinical studies with LKH showed promising results in cancer immunotherapy, namely bladder cancer. The disaccharide epitope Gal(bl-3)GalNAc transversally reacts with an epitope counterpart on the tumor cell. The underlying mechanism is that the compound roots an antibody response in equivalent with an amplification in the activity of NK cells where the humoral and cell-mediated immune response to KLH causes cytolytic reduction in tumor growth. Besides, as a modulator for bladder cancer, KLH and their mucin-like epitopes are extracted to treat colorectal, ovarian, and breast cancer. Likewise, epitopes composed of ganglioside are effective on malignant melanomas (Harris et al. 1997).

10.5.9 Kahalalide F

The depsipeptide compound kahalalide F (KF) was initially extracted from mollusk *Elysia rufescens* but later found it was from the alga *Bryopsis* sp. Studies and experiments showed positive results, both in vivo and in vitro by KF in treatment. KF is a carbon 75 cyclic tripeptide consisting of atypical amino acid Z-dehydroaminobutyric acid (Molinski et al. 2009). The compound is shown useful toward cancer such as breast, colon, non-small-cell lung, prostate, and ovarian (Janmaat et al. 2005). In a few cancer cell lines, KF is reported to cause necrosis like processes.

10.5.10 Naamidine A

Naamidine A produced from *Leucett* sp. a marine sponge is a dibenzylated 2 amino imidazole alkaloid compound that is effective as an inhibitor of epidermal growth factor receptor (EGFR), and the signaling pathway of EFGR has a critical role in the onset of the tumor. Overproduction of EGFR is coupled with the expansion of cells and tumorigenicity in most cancers. Consequently, interference at any stage of the pathway by the compound probably acts as an antiproliferative agent against cancers. Studies showed that ethanol extract is more effective and is capable of

inhibiting EGF-dependent DNA synthesis, mitogenesis, and proliferation. Studies in vivo confirmed the compound hinders the development of squamous carcinoma cell lines (Copp et al. 1998).

10.5.11 Salinosporamide A

Salinosporamide A, a derivative of lactam lactone-γ lactam, is extracted from *Salinispora tropica* in 2003. The compound hinders p26 proteasomes (López-Macià et al. 2001). The compound has passed the first phase of the clinical trial against multiple myeloma (Molinski et al. 2009; Schöffski et al. 2004).

10.5.12 Spisulosine (ES-285)

Spisulosine with a novel structural organization (2S, 3R-2 amino 3 octadecanoic) contributes in parallel to sphingosine-related lipids. The compound ES-285 is produced from *Spisula polynyma,* a mollusk. The cytotoxic effect of ES-285 on cancer cells reveals two different assumptions. The foremost hypothesis is that ES-285 performs as a competitor at the phospholipid growth factor (PLGF). Significant activities such as increased cellular response affecting adherence, chemotactic movement, morphology, survival, proliferation, and ionic conductance are reported by PLGF (98-107). Secondly, the effect of PLGF through endothelial differentiation gene receptors by ES-285 represents a subgroup G protein-coupled receptors, yet the receptors show no role in ES-285 intervene cell termination (Salcedo et al. 2007).

10.5.13 Squalamine

Squalamine extracted from *Squalus acanthias* known as dogfish shark was discovered from New England's shoreline in 1993. The metabolite is an aminosterol with antibiotic activity (Sessa et al. 2002). The Genaera Company was licensed for developing the compound as an antitumor agent against ovarian cancer and solid tumors, which succeeded phase I trials. Studies in clinical and preclinical gave supported squalamine results showing an important role as antiangiogenesis in cancer cells (Pettit et al. 1987).

10.6 Analgesic Property by Natural Marine Compounds

Ziconotide, the first natural analgesic marine drug, was discovered 20 years ago from *Conus* sp., mollusk gastropods. On peptide synthesis, an active compound so named as "ω-conotoxin" is isolated. Nearly 300 species of cone snails synthesize hundreds of peptide toxins affecting the neuromuscular transmission in its organism. However, of all the toxins, the most potent pharmacological toxin used as a drug is

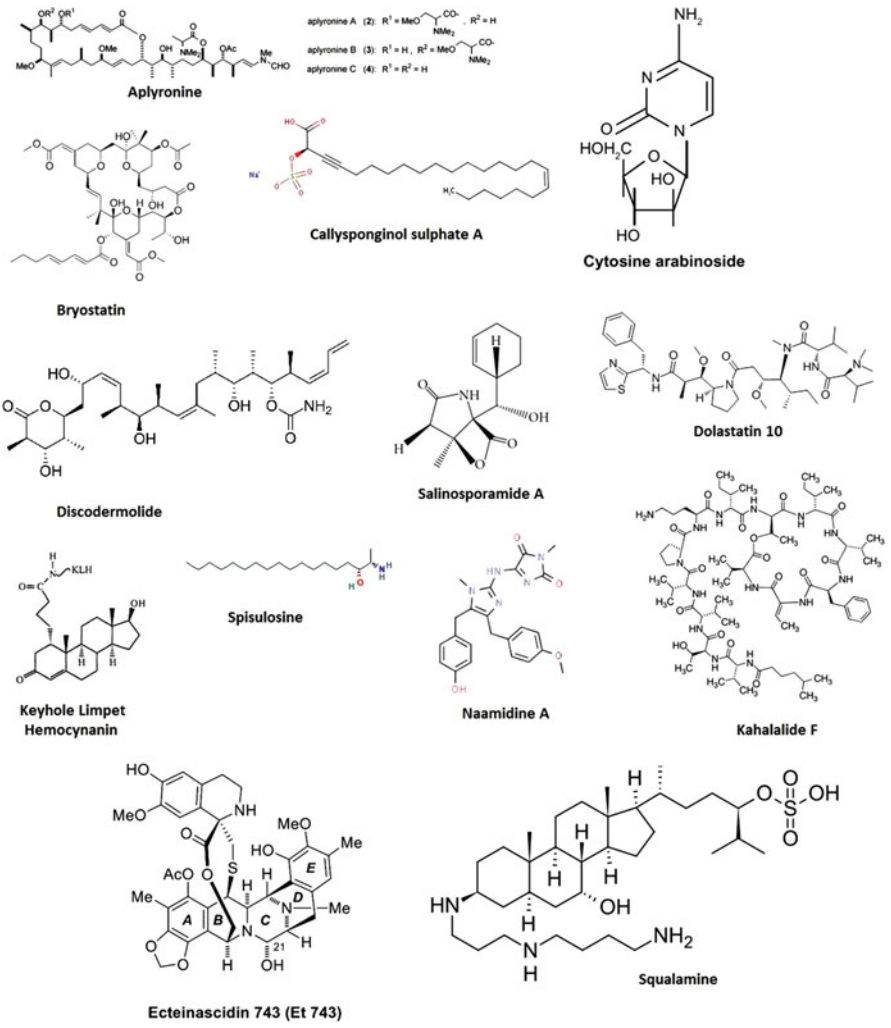

Fig. 10.1 Anticancer compounds derived from marine species

ω-conotoxin MVIIA. The compound is a linear molecular with 25 amino acid residues isolated from *Conus magus*. The drug showed a thousand times higher analgesic response compared to morphine. Clinical studies showed its string effectiveness in hindering pain. Keeping in view the positivity of conotoxins, still investigation is on the path to identifying more other derivatives (46, 47) (Fig. 10.1).

10.7 Immunomodulatory and Anti-Inflammatory Effect by Marine Flora

Marine flora is customized to life in water with large salt absorption but has halogens as their chemical components as oceans are rich in chlorides, iodide, and bromide (Akerkar et al. 2009). The utilization of halogens by marine species results in the modification of their general composition. A surfeit of chemical components has been revealed from these sources. The chemical distinctiveness of marine flora-derived compounds has stepped up the discovery of drugs with a chance of knowing the new molecules and their biological activities (Abad and Bermejo 2001). The most abundant source of bioactive essentials includes alkaloids, antioxidants, oligosaccharides, polyphenols, polysaccharides, steroids, and terpenoids.

10.7.1 Alkaloids

Alkaloids structurally constitute nitrogen molecules with a wide variety of biological activities. They are originated in higher plants and marine species (Dembitsky 2002; Güven et al. 2010). Marine sponges namely *Acanthella aurantiaca* and *Axinella verrucosa* constitute alkaloids that act as inhibitors, especially like NF-κβ (Cimino et al. 1982). Convolutamydine, an oxindole alkaloid isolated from marine bryozoans, is effective in inhibiting the expression of cyclooxygenase-2 (COX-2), prostaglandin-2 (PGN-2), inducible nitric oxide synthase (iNOS), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) in cell lines RAW 264.7, leukocyte migration, and formalin-induced thrashing activities in mice (Fernandes et al. 2014).

Other compounds neoechinulins A and B composed of diketopiperazine indole alkaloid isolated from *Eurotium* sp., marine fungi, exhibit in vitro anti-inflammatory activity lipopolysaccharide-stimulated (LPS) RAW 264.7 cell line. Of both the derivatives, the compound neoechinulin A is safe clinically, but neoechinulin B showed toxicity. Other important mechanisms of neoechinulin A are it downregulates the arrangement or expression of IL-1 and 6, TNF-α, COX-2, PGE2, reactive oxygen species (ROS), and nitric oxide in BV-2 microglial cell oligomeric amyloid, and interrupts nuclear translocation by inhibiting apoptosis. The compound plays an active role in Alzheimer's disease by hindering neuroinflammation (Dewapriya et al. 2013).

Chaetoglobosin Fex (Cha Fex), a cytochalasin alkaloid from *Chaetomium globosum,* represses TNF-α, IL-6, and monocyte chemotactic protein-1 (MCP-1) in RAW cell line and lipopolysaccharide-stimulated peritoneal macrophages. Besides, it reduces the expression of cytokine mRNA, ingress of p65 subunit of NF-κβ into the nucleus, and elevates the levels of p38 and c-Jun. As well, it suppresses the upregulation of CD-14 expression (Dou et al. 2011). In the modern era, the role of alkaloid and alkaloid derivates will play a major role in drug discovery.

10.7.2 Antioxidants

Antioxidants are secondary metabolites that modulate the levels of ROS that roots damage by attaching itself to biomolecules, for instance, DNA. The fundamental principle of antioxidants deactivating ROS-modulating prophylactic and remedial activities for the diseases atherosclerosis, Alzheimer's, cancer, diabetes, inflammatory bowel disorder, Parkinson's disease, rheumatoid arthritis, and stroke is investigated (Behl and Moosmann 2002; Snow et al. 2010; Amaro and Chamorro 2011; Fuchs-Tarlovsky 2013; Ishibashi 2013; Liu et al. 2013a, b). Antioxidants are as an important function as immunomodulators and are utilized in concurrence with conventional therapy in few diseases. They also act as a free radical scavengers; for example, tocopherols reduce the synthesis of PGE-2 and develop cell-mediated immunity (Meydani et al. 1986; Pekmezci 2011). Selenium enhances the phagocytic capacity of macrophages and avoids CD 8+ T-cell damage (Salimian et al. 2014). Marine carotenoids, namely astaxanthin and fucoxanthin, reported having more effectual than terrestrial carotenoids (Guerin et al. 2003; Hussein et al. 2006; Olaizola 2007; Miyashita and Hosokawa 2007). Astaxanthin inhibits the production of induced nitrate oxygen synthase, nitric oxide, and TNF-α in RAW 264.7 cell line (Ohgami et al. 2003). Fucoxanthin hinders inflammatory cytokines IL-1 and TNF-α and reduces the production of antioxidants (Tan and Hou 2014). Iron, selenium, and copper are the most frequent trace elements found in the majority of microalgae (Tenorio-Rodríguez et al. 2013). Both microalgae and seaweeds have high levels of ROS, but due to changes in environmental conditions, the levels of ROS in seaweeds are altered as the seaweeds neutralize by the high intracellular quantity of antioxidant compounds, namely carotenoids, phycobilins, polyphenols, and vitamins (Cornish and Garbary 2010).

10.7.3 Polyphenols

Marine flora is a good source of polyphenolic compounds such as anthocyanins, catechin, epicatechin, epigallocatechin, flavonoids, tannins, lignin, and phlorotannins (Harborne and Williams 2000; Bravo 1998; Cheynier 2005). Polyphenol compounds do have great bioactivities, for instance, cardiovascular defender, antitumor (Andriantsitohaina et al. 2012; Middleton et al. 2000; C Recio et al. 2012), anti-inflammatory, immunomodulatory (Puupponen-Pimiä et al. 2001; Giovannini et al. 2007), and antioxidant activities (Heim et al. 2002). The significant mechanism of polyphenols in cancer and inflammation activities is the cell signaling and regulation of genes by modulating the NF-κβ pathway. Besides, it downregulates activities such as transcription, phosphorylation, and expression (Sun et al. 2006; Holmes-McNary and Baldwin 2000; Adhami et al. 2003; Manna et al. 2000). A phlorotannin compound, diphlorethohydroxycarmalol (DPHC), isolated from *Ishige okamurae,* exhibits anti-inflammatory activity by hindering IL-6 production effectively in lipopolysaccharide RAW 264.7 cells. Besides, DPHC amplifies the production of suppressor of cytokine signaling by inhibiting the transducer expression

activity and transcription 5-activator signaling (Kang et al. 2015). Another phlorotannin extracted from *Fucus distichus* decreases the expression of IL-10, MCP-1, TNF-α, and COX-2. Nevertheless, phlorotannin acts as modulators in inflammatory signaling, namely Toll-like receptors and downstream molecular pathways of NF-κβ, p38, and JNK (Kellogg et al. 2015).

10.7.4 Polysaccharides

The most abundant and chemical complex organic molecules in the oceans are polysaccharides with variable therapeutic activities with no or less toxicity (Laurienzo 2010). Marine flora, mainly algae and bacteria, is a common source of polysaccharides. Sulfated polysaccharides increase the innate immune response by upholding the macrophages and NK cell tumoricidal activity (Yim et al. 2005; Gorelik et al. 1984; Gorelik 1987; Zhou et al. 2005). Polysaccharides derived from both micro- and macroalgae have anti-inflammatory activity (Cumashi et al. 2007; Lüscher-Mattii 2000), the most common be fucoidans extracted from brown seaweeds with immunomodulation property (Li et al. 2008; Jiao et al. 2011). Another sulfated polysaccharide p-KG03 isolated from *microalgae Gyrodinium impudicum* strain KG03 enhances the production of nitric oxide in the c-Jun-N-terminal kinase pathway activates the production of cytokines IL-1 and IL-6 and TNF-α in macrophages and averts the development of cancer cells both in vivo and in vitro (Bae et al. 2006). A novel colloidal polysaccharide, alginic acid isolated from brown seaweed, hinders the secretion of cytokines IL-1 and TNF-α (Jeong et al. 2006).

Exopolysaccharide (EPCP1-2) isolated from *Crypthecodinium cohnii* show anti-inflammation property by downregulating Toll-like receptor 4 pathway (Kinnel et al. 2017). Marine bacteria especially the habitat of Antarctic, hydrothermal vents, and hypersaline lakes are a source of exopolysaccharides (Nicolaus et al. 2010; Guezennec 2003; Maugeri et al. 2002; Poli et al. 2010; Gugliandolo et al. 2012). EPS1 extracted from haloalkaliphilic thermophilic *Bacillus licheniformis* strain T14 inhibits herpes simplex virus-2 replication in adult human peripheral blood mononuclear cells. The mechanism shows a host defense activity against viruses by enhancing the immune response (Gugliandolo et al. 2014).

10.7.5 Steroids

Steroids are cholesterol-derived lipophilic metabolites found from earthly, marine, and human-made sources. Steroids and their components play a significant role in biological activities such as contraceptives (Lopez et al. 2014), anticancer agents (Thao et al. 2015), antiasthmatic anesthetics, a contender for hormones (Jovanović-Šanta et al. 2015), cardiovascular therapeutic agents (Rattanasopa et al. 2015), anti-inflammatory (Aav et al. 2005), osteoporosis treatments (Cortet et al. 2011), and antibiotics.

Modulation of pregnane X receptors (PXR) in lowering the intestinal inflammation by solomonsterol A extracted from marine sponge *Theonella swinhoei* is one example of a steroid compound having a significant role in living organisms (Fiorucci et al. 2012; Sepe et al. 2011). Solomonsterol A also hinders the growth of arthritis caused due to anticollagen antibodies in genetically engineered mice wharfing PXR2. Solomonsterol A prevents the expression of inflammatory markers, cytokines, and chemokines, which decrease the inflammatory response (Mencarelli et al. 2014). Pregnane steroids isolated from soft coral *Seleronephthya gracillimum* decrease the accumulation of the pro-inflammatory proteins COX-2 and iNOS.

Another example of steroid compounds is the ergosta-7, 22-dien-3-ol isolated from *Marthasterias glacialis* commonly known as spiny sea star that exhibits anti-inflammatory property in RAW cell lines by downregulation inflammatory markers. *Astropecten polyacanthus*-derived steroid compound showed downregulation for the production of cytokines in the LPS bone marrow of dendrites (Thao et al. 2013). Michosterols A to C structurally depicting polyoxygenated steroids isolated from *Lobophytum michelle,* the soft coral, showed the effective anti-inflammation property in restorative neutrophil production of superoxide anion and liberation of elastase in N-formyl-methionyl-leucyl-phenylalanine/cytochalasin B (Wang and Miao 2013).

10.8 Bioactive Compounds as Modulators for Bone Growth and Wound Healing

Osteoporosis and related fractures are one of the leading problems in European countries where fractures take place once every three seconds (Johnell and Kanis 2006). Fractures are most common in postmenopausal women and are a lot hurting, and have the low healing capacity (Hernlund et al. 2013). Modern treatment is imperfect as they are not efficient and are recommended for a longer period. Though drugs have been in use, they are reported to be effective only in lowering the fracture risk but do not increase the bone density besides many side effects (Ensrud et al. 2004). Apart from the cellular factor, marine species are a potent source of scaffold material and propping up new osteoblast bone development (osteogenic). Few marine-derived compounds with osteogenic effects cultured in vivo are compound sargachromanol G extracted from *Sargassum siliquastrum* (Yoon et al. 2013), quinone derivatives isolated from *Sargassum thunbergii* (Kim et al. 2016), water molecule as by-product from *Hizikia fusiforme* (Jeong et al. 2016), crude extract of *Cladophora rupestris* and *Codium fragile* (Surget et al. 2017), compounds floridoside (Ryu et al. 2015) and aquamin (Ryan et al. 2011) isolated from *Laurencia undulata* and *Lithothamnion corallioides*, macrolide derived from *Lyngbya* sp. (Yonezawa et al. 2012), symbioimine produced from *Symbiodinium* sp. (Kita et al. 2004), a polyketide from *Amphidinium* sp. (Minamida et al. 2014), peptide and polysaccharide from *Nannochloropsis oculata* and *Alteromonas infernus,* respectively (Nguyen et al. 2013; Merceron et al. 2012), largazole depsipeptide extracted from cyanobacteria *Symploca* sp. (Lee et al. 2011), phorbaketal A from marine

sponge *Phorbas* sp.(Byun et al. 2012), norzoanthamine from zoanthid (Kinugawa et al. 2009), coral cell extracts from hard and soft corals namely *Xenia elongate* and *Montipora digitata* (Helman et al. 2008), fucan sulfate extracted from *Apostichopus japonicus* (Kariya et al. 2004), proteins from Abalone especially perlucin (Weiss et al. 2001), and adhesive (Hong et al. 2012) extracted from *Haliotis* sp. Nacre, a water-soluble protein, was produced in oysters *Crassostrea gigas* (Oliveira et al. 2012) and *Pteria martensii* (Kim et al. 2012) and *Pinctada margaritifera* (Bédouet et al. 2007).

Marine natural products act as modulators in wound healing. Trepangs (sea cucumbers) of *Holothurians* are reported to have healing properties along with stimulation. But, the compounds liable for the bioactivities of trepangs are yet to be known. Collagenase KK, a complex drug extracted from *Paralithodes chamtschaticus,* is suggested for wound healing by the Russian institute. The drug showed effective for chronic osteomyelitis, bone cavities, chilblains, necrosis, ulcer in the varicose region, and gangrene (Kozlovskaya et al. 1997; Stonik et al. 2007; Elyakov and Stonik 2003). Collagenase KK has potent applications in other therapeutic fields such as in postsurgery complications, for example, in plastic surgery and endoscopy, and in other diseases unlike treating the children with fetid peritonitis. The compound also is meant for the devastation of collagen in the brain commissures. Clinical studies of methopterosin, a synthetic derived of pseudopterosin, revealed anti-inflammatory activity against contact dermatitis. However, the trial was disrupted. But later, the compound was assessed to the second phase of clinical testing reported potential activity for healing wounds (Gross and König 2006).

10.9 Antimicrobial Effect of Marine Flora as Immunomodulators

From the past 10 years, studies have focused and challenged for natural marine compounds stimulating antimicrobial activity. Investigations reported marine species extracted from algae, bacteria, fungi, and sponges; tunicates produce novel compounds with an antibacterial property that helps in treating drug-resistant bacterial infections. Researching on antifungal compounds, few studies reported novel products from marine fungi, sponges, algae, and sea cucumbers delineated. Table 10.3 illustrates novel marine compounds with antibacterial and antifungal activities.

10.10 Other Immunomodulation Activities of Marine Flora

Extensive studies of marine flora having anthelmintic activity are in practice. A recent study reported a triterpene glycoside compound echinosides A and isolated from *Actinopyga echinites* and *Holothuria poli* belonging to marine flora commonly

Table 10.3 Antimicrobial activity and mechanism of marine-derived compounds

Compound	Source	Chemistry	Pharmacological activity	Drug effect	References
Anthracimycin	Actinomycetes	Polyketide	The compound is effective against *Staphylococcus aureus* and *Bacillus anthracis* by inhibiting DNA or RNA	Antibacterial	Jang et al. (2013)
Aflatoxin	*Aspergillus flavus*	Polyketide	Hinders *B. subtilis* and *E. aerogenes* but activity is undetermined	Antibacterial	Wang et al. (2012a, b, c)
Ageloxime	*Agelas mauritiana*	Alkaloid or Terpenoid	Inhibits the growth of *S.* aureus. The mode of action is yet to determined	Antibacterial	Yang et al. (2012)
Chrysophaentins	*Chrysophaeum taylori*	Shikimate	Competitive inhibition of gram-negative and positive bacteria by binding to FtsZ GTP site	Antibacterial	Keffer et al. (2013)
Merochlorin a	Actinomycetes	Terpenoid	Effective against *C. difficile* and *S. aureus* by obstructing synthesis of DNA, RNA, protein, and cell wall arrangement	Antibacterial	Sakoulas et al. (2012)
Altersolanol c, macrosporin, alterporriol	*Alternaria* sp.	Polyketide	Shows inhibition activity for species *E. coli* and *V. parahemolyticus*	Antibacterial	Zheng et al. (2012)
Axistatins 1-3	*Agelas axifera*	Alkaloid/ terpenoid	Hinders *S. aureus* and *C. neoformans*	Antibacterial	Pettit et al. (2013)
Bromophycoic acid A and E	*Callophycus* sp.	Diterpene-benzoate (terpenoid)	Inhibits *S. aureus* and *E. faecalis growth*	Antibacterial	Teasdale et al. (2012)
Cadiolides C–F	*Pseudodistoma antinboja*	Shikimate	*S. aureus* inhibition	Antibacterial	Wang, Kim et al. (2012)
Ageloxime B	*Agelas mauritiana*	Alkaloid/ terpenoid	The compound is effective in inhibiting *C. neoformans*	Antifungal	Yang et al. (2012)
Aurantoside K	*Melophlus* sp.	Polyketide/ alkaloid	Hinders the *C. albicans* mechanisms	Antifungal	Kumar et al. (2012)
Caulerprenylol B	*Caulerpa racemosa*	Glycoside	*C. glabrata* and *C. neoformans* inhibition	Antifungal	Liu et al. (2013a, b)

Crambescidin-816	*Crambe crambe*	Alkaloid	Hinders *S. cerevisiae* growth by inducing cell cycle arrest at G2 or M phase before apoptosis and mitochondrial disfunction	Antifungal	Rubiolo et al. (2013)
Didymellamide A	*S. cucurbitacearum*	Alkaloid	Hampers the growth of *C. albicans*	Antifungal	Haga et al. (2013)
Hippolachnin A	*Hippospongia lachne*	Polyketide	The compound is effective against *T. rubrum, M. gypseum,* and *C. neoformans*	Antifungal	Piao et al. (2013)
Holotoxins F and G	*Apostichopus japonicus*	Terpenoid	Obstructs the growth of *C. albicans, Microsporum,* and *Cryptococcus* and avoids fungal infection mode of action that is yet to be determined	Antifungal	Wang and Miao (2013)
Hyrtimomine D and E	*Hyrtios* sp.	Alkaloid	*C. albicans* and *C. neoformans* growth inhibition	Antifungal	Tanaka et al. (2013b)
Nagelamide Z	*Agelas* sp.	Alkaloid	inhibition *C. albicans* growth	Antifungal	Tanaka et al. (2013a)
Neothyonidioside	*Australostichopus mollis*	Terpenoid glycoside	*S. cerevisiae* inhibition by disturbing membrane curvature and fusion necessary for membrane reprocessing and lysosomal degradation	Antifungal	Yibmantasiri et al. (2012)
Woodylide A	*Plakortis simplex*	Polyketide	*C. neoformans* growth inhibition	Antifungal	Yu et al. (2012)

known as sea cucumbers. The compounds showed promising results against *Schistosoma mansoni* worms (Melek et al. 2012).

A novel phenolic compound, octaphlorethol A extracted from *Ishige foliacea* a marine alga, has antidiabetic activity. The mode of action is the enhancement of glucose uptake by escalating glucose transporter 4 translocations to the plasma membrane, AMP-activated kinase, and protein kinase B activities (Lee et al. 2012).

A new-fangled compound lobocrassin B extracted from *Lobophytum crassum* showed immunomodulatory effects on dendritic cells of bone marrow. Chemically, the marine product belongs to membrane type diterpenoid. Lobocrassin B is shown to soothe dendritic cell maturation and activation with simultaneous inhibition of TLR-stimulated translocation of inflammatory cytokine production and the protein complex NF-κβ. Lobocrassin B has a therapeutical role in immune dysfunctions (Lin et al. 2013). Another novel marine-derived compound penicacid B produced from *Penicillium* sp. obstructs splenocyte lymphocyte proliferation by hindering inosine 5-monophosphate dehydrogenase enzyme in purine synthesis. The significant role of penicacid B is its immunosuppressive activity. The compound is structurally mycophenolic acid derivative (Chen et al. 2012; Jensen et al. 2012).

Preclinical studies on marine nervous system pharmacology reported potential modulatory activities especially focusing the membrane channels sodium and potassium, and receptors such as nicotinic acetylcholine, and neuroprotection, analgesia, and antinociception. Few peptide derivatives isolated from varied marine flora show potential results on the nervous system by inhibiting different mechanisms happening within the system. Few important compounds are APETx2 and BcsTx isolated from the sea anemone, asteropsin A from the sponge, and convolutamydine A from bryozoa. APETx2 inhibits acid-sensing ion channel 3 (ASIC3) by reducing the potency in opposition to non-voltage-gated and truncation at either C or N terminal, affecting the binding activity of ASIC3 (Jensen et al. 2012). BcsTx derivatives 1 and 2 isolated from *Bunodosoma caissarum* confirmed inhibition of potassium influx activity (Favreau et al. 2012, Vetter et al. 2012). A steopsin A obstructs binding with voltage-gated sodium channel site 2 and includes drug development of cysteine knot peptide-based as a mold gibbet (Li et al. 2013). Few other alkaloid compounds from marine sponges namely ianthellamide A (Feng et al. 2012), leucettamine B (Burgy et al. 2013), and pulchranin A (Guzii et al. 2013) have shown probable activity by inhibiting kynurenine 3-hydroxylase for enhancing kynurenic acid in vivo, tyrosine phosphorylation kinase inhibition (reduces neurodegeneration), and inhibition of transient receptor potential cation channel subfamily V member 1, respectively. Inhibition of acetylcholinesterase by terpenoid compounds arigsugacin I and asperterpenol A isolated from marine fungus showed probable activity toward the nervous system (Huang et al. 2013; Xiao et al. 2013). Few other terpenoids halomadurones C and D, ircinianin lactams, and polar steroids showed effective to the nervous system, but the mechanism is yet to be known (Wyche et al. 2013; Balansa et al. 2013; Palyanova et al. 2013). Cymatherelactone, a polyketide isolated from marine alga, reported to hinder voltage-gated sodium channel but the mechanism of action is yet to be determined (Choi et al. 2012).

10.11 Challenges and Future Perspectives

The aquatic environment is habitat to an enormous number of micro- and macroorganisms with unexploited biological activities that are used to a greater level to make sure their existence in this diverse and regular hostile home. This unique environment makes possible the biosynthesis of a range of secondary metabolites to act as a chemical defense and show a wide array of bioactivities. However, the marine environment is susceptible to variation in sea levels and acidification due to the rise in carbon dioxide levels as the climatic varies, where a majority of species cannot adapt to these changes. This causes a serious issue to the world as marine natural products are the neediest compounds in preventing and treating diseases. So, to continue oceans maintaining or sustaining us, we ought to discover a means to maintain them. To overcome these effects, few interventions should be exploited through effective functioning by the government organizations, individuals, and communities to restore and protect the habitats. Additionally, it is the responsibility of the man to downregulate pollution and overdevelopment of industries at the oceanfront, which reports negative impact leading to changes in the complex ecosystem.

Though the ocean contains a large source of functional organisms, the majority of these areas are still unreachable to the scientists. To get the ease of inaccessible regions, both researchers and oceanographers are to have a good understanding. Certain developments are required to get promising results. One such way is the support systems to the underwater life, which should be exploited in an improved way. Marine flora is a chemically structured, having novel potent compounds that act as an effective drug against diseases.

To succeed biological assays such as finding out the target site, mode of action, compound selectivity, and its cytotoxicity, bulk amount of a compound is necessary. Likewise, for preclinical and clinical trials, several hundred grams to kilograms of the compound is essential to know the exact functioning and application of the specific compound. This is one of the major tailbacks for the researchers to develop marine natural products for clinical applications. To overcome these problems, synthetic chemists are developing artificial strategies to get better results in bringing these compounds to the preclinical phase and finally to get it into the commercial and medicinal field. Marine natural compounds are capable to be chemically customized with different steric structural elements to enlarge "drug-like" compounds. As well, mariculture and aquaculture have been challenged to resolve the hitch of sustainable delivery of macroorganisms. Yet, the distinctive and at times selective conditions of the sea formulate development or maintenance of extracted samples is still very challenging and habitually impractical. For the preclinical phase trails to develop customized marine natural product, it strains complex, mechanistic, and pharmacokinetic studies, and those itself is a massive challenge yet nonetheless stimulating task. From a large-scale perception, the marine pharmaceutical channel remains active and currently appears to have enough thrust to bring the additional compound to the souk in the coming days. The functionality of various compounds against

pathogens is extremely encouraging, yet the development and application will continue with extreme hope.

Several techniques are in practice to get the best from the marine environment. Genome mining has accompanied in a resurgence in the field of natural product inventions, giving new expectations in the unending search for novel marine products. This stratagem lets researchers strap up the factual biosynthetic perspective, which dwells within varied groups of marine flora and suggests an important imminent not only for the biosynthetic activities but also for the evolutionary and self-protective strategies the organism makes use of in the marine environment. To make sure and possible for the marine products to reach the marketplace, collaborative events, including various disciplines such as general chemistry, organic and medicinal chemistry, biology, pharmacology, and bioinformatics, should get associated. For the betterment of drug improvement, a biological technique transferring genetic material in the host cells from the preferred compound is under development. This is a significant field for controlling the isolated and expressed genes of the marine species, which would assist us to have an added targeted loom in developing compounds from the marine source. Compounds extracted from marine macroorganisms are sometimes difficult to develop separately from cultures because few organisms are in symbiotic association with the host cell, where the desired genes through cultivated in vitro do not seem expressive. For such samples, metagenome analysis is an apt technique, which is still under development. Another major challenge is the compound's availability in an adequate amount to be utilized for clinical trials. Other few drawbacks are the ecological viability and cost feasibility essential for stirring up a structural modification to increase the drug properties.

Regarding the search for novel antiviral agents, the research is still ongoing so that the discoveries may lead to getting more potent and new-generation drugs to fight against viral diseases in humans and other animal species. For the production of these compounds, assimilation linking molecular modeling designs and combinatorial biochemistry alongside postgenomic tools perhaps be used for continuous production. By now, many marine compounds are being analyzed clinically and are produced either by chemical synthesis, aquaculture, or fermentation practices (Munro et al. 1999). Though chemical synthesis is apt for some metabolites, it might be cost-effectively not feasible for others (Bhadury et al. 2006). A new challenge with antiviral drugs is the resistance attenuation by diverse viruses. With numerous unrevealed organisms with inimitable metabolites in the marine habitats, a rising number of novel drugs may be revealed that viruses have not still developed resistance. However, it is imperative to note that several organisms of varied species generate the same classes of compounds, each one suitable for their distinctive composition and existing habitat. Though producing varied derived common categories of the compound by multiple organisms is a solution, a virus that resists to a specific drug might not defiant to other naturally occurring derivatives, which have the prospective to own alike, if not the same, antiviral activities. The application of biochemical tools will let the exploitation of occurring marine natural compounds generate an imitative chemical extremely advanced to that of the original. This leads to the establishment of metabolites with lower cytotoxicity and

improved specificity. So, the discovery of novel drugs and alteration of modern drugs will play a significant role in the fight in opposition to viral resistance. Advance technologies such as structure elucidation by nanoscale nuclear magnetic resonance, sampling techniques, total chemical synthesis, and biosynthesis by whole-genome sequencing and genetic engineering are fundamental tools to achieve marine natural products as drugs. Moreover, as stated above, modification of the culture conditions may pave the way for changes in the metabolic field. The drug industries should ponder on how aptly they should maintain the physicochemical factors such as optimum pH and temperature, the quantity of oxygen accessible, and evade variation of secondary metabolites. Besides in vitro studies, in vivo studies should be carried to pass the preclinical trials with impending results for therapeutic purposes.

A better understanding of the microbial physiology, systematics, metabolism, and sequencing multiple genomes of actinomycetes is essential as it is the most common distribution in the marine environment with varied functional activities and one of the productive sources in generating commercial compounds. Rather than general culture techniques, advanced tools such as high-throughput cultivation are an inventive method that ape nature and smooth the progress of growth of actinomycetes by reducing unwanted, fast-growing bacteria, because actinomycetes are a slow cultivator.

The future endeavors for marine natural products as a potential modulator should involve sound indulgent, especially to the microbial flora. Ten specific tips are taken into account include:

1. Augmented information of microbial ecosystem and physiology with an ecophysiological point of view, i.e., the distinctiveness of environmental substrates that control the growth of microorganisms.
2. Expansion of more intended, less conservative isolation methods, concerning mainly about the functional diversity, and physiological characters of native organisms.
3. Better understanding and attention in the learning of unknown or farthest environments.
4. Advanced technologies like matrix-assisted laser desorption ionization–time-of-flight (MALDI-TOF) mass spectrometry to identify preconditions.
5. Exploitation of latest techniques, for example, heterologous expression, gene interruption, and bioinformatics.
6. Utilization of taxa-related subjects such as chemical taxon and taxon features as a result that the compounds to facilitate openly implicated in the synthesis of secondary metabolites and could be used to get data about the biodiversity of antibiotics generated from varied organisms.
7. Developing advanced technologies in the area to increase the exploration range of natural products isolated and effectual deduplication.
8. Proposing high-throughput effectual screening strategies.
9. An innovative insight of polyphasic loom and effective computational investigation with genomic data will provide considerable information to disclose the site

and chemical taxon characteristics of earlier undetected bioactive actinomycetes. This would certainly direct in the direction of new invention and utilization of novel metabolites with extensive and significant contributions. According to our review, the greater extent of innovation to discover novel drugs and their development gives justification for our hopefulness that natural marine compounds will shape a new gesture of medicines that release into the marketplace and pharmacies in upcoming days.

10.12 Concluding Remarks

Regardless of the prospective for unearthing new drugs within this field, the present research is said to be extremely narrowed. In contrast to the vast diversity of marine species, and only a small part of them have been analyzed for their biological activities, and yet smaller numbers expressly for antimicrobial, bone disorders, and antiviral and cancer therapies. Most secondary metabolites isolated originate from metabolic processes at exertion in microorganisms, especially actinomycetes, cyanobacteria, microalgae, and fungi, and logically, it is providential for the biosynthetic pathway that code for natural compounds in prokaryotes is well-known and more acquiescent for research study than the eukaryotes. Additional research is essential so that the studies cover all the taxonomic clusters. Though both in vitro and in vivo studies have detailed general activities of the marine novel metabolites, the mode of action or the mechanism of activity and the biochemistry of the few compounds are unclear. Though we are into the twentieth century where trials on humans are still rare, studies should get advanced and much focused in the future so that we achieve promising results within the field. In conclusion, marine metabolites are very promising with varied biological activities and potent toward therapeutics and pharmacological effects. The shortage of research in this field increases discovery probability, making it an exhilarating area to carry out investigations. Better study attempts might direct to new inventions for effectual therapeutic alternatives, whereby will directly reduce the burden on the healthcare system and enhance the standard of health in patients.

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Marine Origin Bioactive Peptides: Novel Advances in the Therapeutic Potential

11

Mohsen Dehghani, Mohammad Reza Taherizadeh, and Ahmad Homaei

Abstract

Marine organisms contain valuable chemical compounds as secondary metabolites such as bioactive peptides that survive in harsh marine environments produced by organisms. They have been considered by scientists for use in medicine and pharmaceutical researches due to their unique and unknown biological properties. Studies on a variety of marine-derived peptides have shown that these compounds as antimicrobial, antiviral, antioxidant, antihypertensive, and anticancer agents can be useful in the treatment of various diseases. While many marine peptides are in various phases of clinical trials, two marine peptide-derived drugs have been approved by the Food and Drug Administration (FDA), too. This chapter highlights the recent two decades of researches on the biological properties of peptides derived from a variety of living organisms, including microbes, plants, and marine animals, in vivo, vitro, and clinical trials and their potential for disease treatment.

Keywords

Marine bioactive peptides · Biological activities · Enzymatic hydrolyze cytotoxicity · ACE inhibitor · Clinical trials

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11.1 Introduction

The marine environment covers about 70% of the earth's surface (Jo et al. 2017) and containing about 50% of living organisms (Barbosa et al. 2020). Marine organisms produce many bioactive natural products because they are subject to a variety of environmental stresses including changes in water chemical composition, temperature fluctuations, currents, and storms, and due to food and space constraints, they must use different strategies to survive (Aneiros and Garateix 2004; Buijs et al. 2019). One of these strategies is to produce and use unique chemical compounds, named as secondary metabolites, to deal with stresses (Buijs et al. 2019).

In contrast to primary metabolites (e.g., lipids, nucleic acids, amino acids, carbohydrates), secondary metabolites (have low molecules weight, diverse chemical structures, and biological activities) are not necessary for growth and reproduction but are crucial for survival (Mosunova et al. 2020). Peptides are important bioactive secondary metabolites, which are produced by marine organisms for biological goals (Cheung et al. 2015). Bioactive peptides are composed of 2–20 amino acid residues and based on composition and sequences of their amino acids show different bioactive behaviors, and have been extensively studied because of their significant bioactivities (Fan et al. 2014; Lee et al. 2017). Using improving sampling techniques and different separation ways can gain marine bioactive peptides (Jiménez 2018), so that currently, over 2,140,000 secondary metabolites are known (Thirumurugan et al. 2018), of which 20,000 are marine compounds, of which about 2000 are peptides (Giordano et al. 2018). Release of bioactive peptides is carried out by three techniques including solvent extraction, enzymatic hydrolysis, and microbial fermentation (Fan et al. 2014), but marine bioactive peptides are mostly isolated by enzymatic hydrolysis of proteins (Kim and Wijesekara 2010; Lee et al. 2012). Marine bioactive peptides due to key roles in different physiological mechanisms such as depression, stimulation, and inhibition appear to be useful in biomedical and pharmacological researches (Aneiros and Garateix 2004; Bashir et al. 2020; Cheung et al. 2015; Ghanbari 2019; Harnedy and FitzGerald 2012; Sánchez and Vázquez 2017).

Researchers over the two recent decades have paid special attention to marine bioactive peptides as novel natural product in context of human health and their effects of therapy or reduce various human diseases (Kang et al. 2018; Kobayashi 2016; Ovchinnikova 2019). They may have positive effects as anticancer (Anand et al. 2019; Kang et al. 2018; Karpiński and Adamczak 2018), antimicrobial (Boto et al. 2018; Oh et al. 2020), antibacterial and antifungal (Ennaas et al. 2015; Hwang et al. 2019), antiviral (Ma et al. 2017a, b), antibiotic (Kormilets et al. 2019), antihypertensive (Je et al. 2020; Sun et al. 2019a, b), antioxidant (Sila and Bougatef 2016); (De Domenico et al. 2019), anticoagulant (Jo et al. 2008), antitubercular (Hou et al. 2019), immunomodulatory (Kang et al. 2019), and other therapeutic agents. However, due to difficult access to marine environments and collecting marine organisms (Martins et al. 2014), probably many natural chemicals, especially bioactive peptides, are unknown and must improve the diving instruments and collecting tools of marine organism, especially in depth waters. This chapter provides an

overview of recent studies on marine bioactive peptides derived from various marine organisms including marine bacteria/fungal/cyanobacteria, algae, sponges, mollusks, fishes, and other marine micro/macro-organisms, and their bioactivities on the disease agents in vivo and in vitro models and clinical trials and action mechanisms and finally introduces the marine bioactive peptide-based pharmaceuticals that have been approved for marketing.

11.2 Methods for the Isolation of Bioactive Peptide

There are generally three groups of bioactive peptides: (1) active peptides that are extracted directly from marine organisms; (2) peptides that are produced by various enzymes using hydrolysis of proteins from marine sources; and (3) peptides obtained from the fermentation product of microorganisms (Falkenberg 2014) (Fig. 11.1). Isolation of peptides from fermentation is not common, so only the isolation

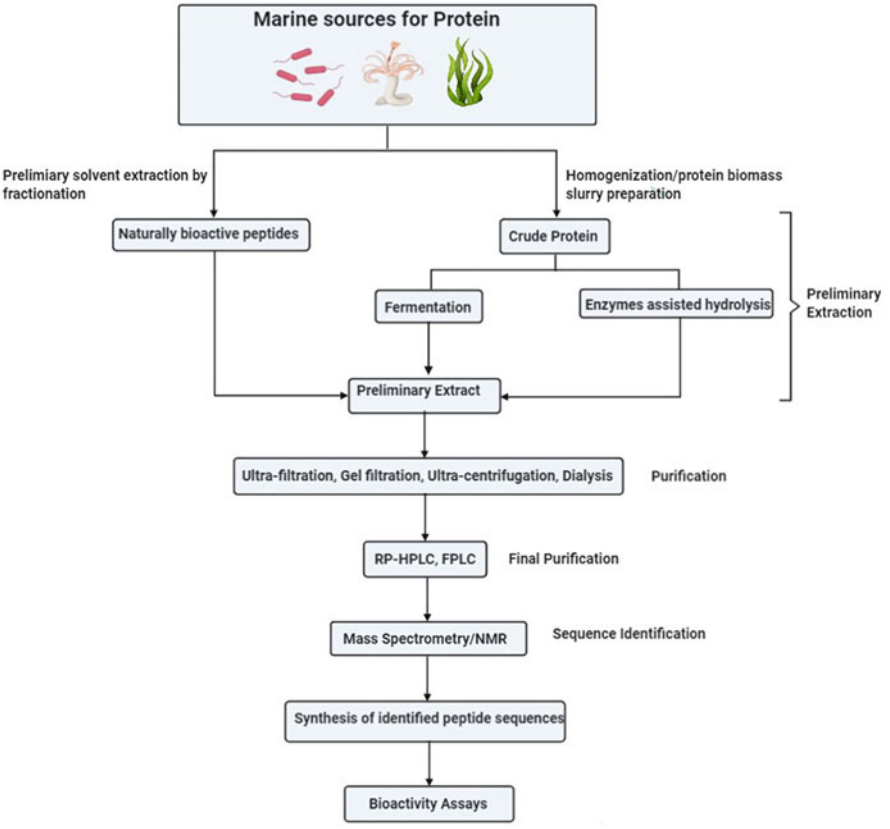

Fig. 11.1 A schematic view of the general isolation and purification procedure for marine bioactive peptides

methods of the first and second groups are explained. Various methods such as ultrafiltration, microfiltration, reverse osmosis, nanofiltration, gel filtration, and ultracentrifugation are used to separate the large proteins from residual particles (Fig. 11.1) (Lemes et al. 2016). The isolated peptides are prepared for the next steps to obtain pure peptides using various purification techniques such as ion-exchange chromatography, gel permeation, reversed-phase high-performance chromatography, and reversed-phase high-pressure liquid chromatography (RP-HPLC) (Cheung et al. 2015; Sable et al. 2017) (Fig. 11.1). As well as, using advanced mass spectrometric techniques such as matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI–TOF–MS/MS) or NMR spectroscopy, the amino acids and sequences of peptides are identified (Sable et al. 2017) (Fig. 11.1). The first group of the above bioactive peptides can be isolated directly from the biomass using common solvents such as methanol and acetone (R. C. Cheung et al. 2015) (Fig. 11.1). Then, in the fractionation stage, the crude extract is exposed to various gradient systems including low and high polarity solvents, and medium polarity gradient solvents such as dichloromethane, tetrahydrofuran, and ethyl acetate to the peptides remain in solution (Ebada et al. 2008). HighAQ5-performance or reverse-phase chromatography (RP-HPLC) methods are used for more purification of these solutions, so that this technique is named bioassay-guided (Aneiros and Garateix 2004) (Fig. 11.1). In the enzymatic hydrolysis, bacterial or fungal plant enzymes such as alcalase, pepsin, trypsin, flavourzyme, and protease are used to hydrolyze the parent proteins, and after hydrolysis, the extracts are exposed to gel filtration and reversed-phase HPLC, and amino acid sequences are analyzed by MALDI-TOF/TOF MS/MS (Sable et al. 2017).

11.3 Marine Organism Peptides

11.3.1 Marine Bacteria

Marine microorganisms including protest, fungi, bacteria, and viruses comprise 90% of weights of all organisms present in the ocean, so that there are 102 fungi, 103 bacteria, and 107 viruses in one millimeter of seawater (Agrawal et al. 2017). Marine bacteria, as chemical gold, are important source of new medicine and drug sciences, and natural products of marine bacteria are the wealth of the sea. Meanwhile, several groups of marine bacteria are coexisting with many species of marine organisms, especially invertebrate (Joseph 2017) whose natural products are potential in pharmacology (Piel 2006). Fifty percent of clinical drugs are belonged to nonribosomal peptides that 70% of which are marine microbe peptides (Agrawal et al. 2017). Most of the articles reviewed below are about the antimicrobial and anticancer properties of bacteria.

Bioassay of new thiopeptide, TP-1161 purified from marine Gram-positive bacterium *Nocardiopsis sp.* (collected from marine sediments and sponges of Trondheim Fjord, Norway), was valued in vitro, and the results showed a potent inhibitory activity versus the growth of vancomycin-resistant *E. faecalis* 560 and

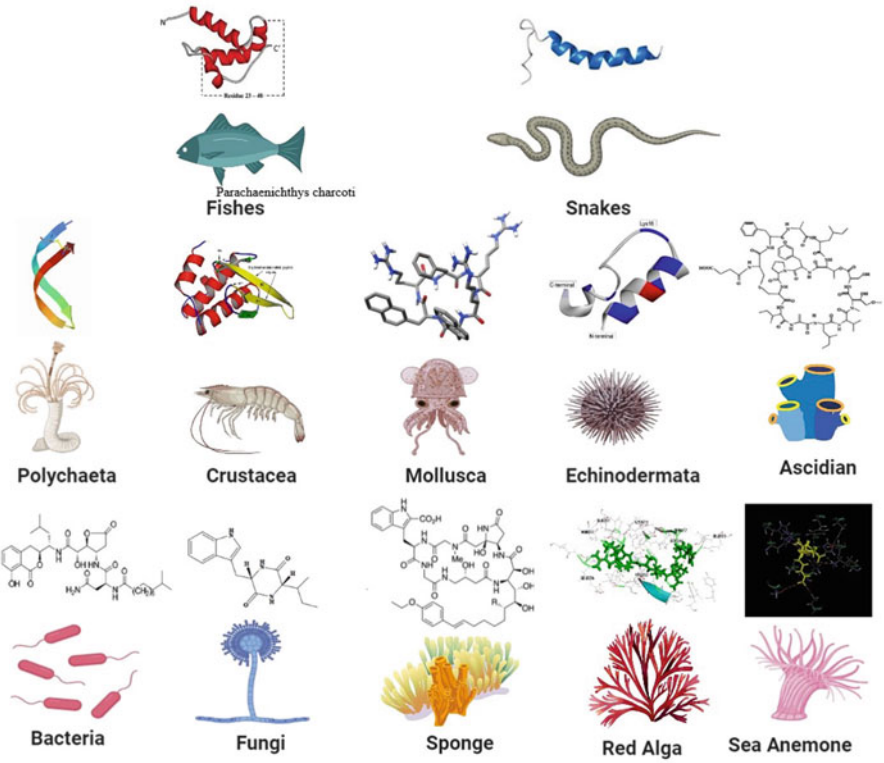

Fig. 11.2 Types of marine bioactive peptides extracted from the different marine organisms and their structures

vancomycin-resistant *E. faecium* 569, so could be used as antibiotic drug in clinical researches (Engelhardt et al. 2010). From marine sediments of Fiji, a Streptomyces sp. was sampled and the bioactivity assessment of its desipeptides named fijimycins A-C and etamycin was tested. These peptide exception Fijimycins B displayed high anti-MRSA activity versus three MRSA strains (ATCC33591, Sanger 252, and UAMS1182) (Sun et al. 2011). Peptidolipins B-F are five new lipopeptides that were isolated from marine Gram-positive bacteria. Two of these peptides (e.g., type B and type E) showed antibacterial effects versus methicillin-sensitive *S. aureus* (MSSA) and MRSA, while peptidolipins C, D, and F observed weak activity against MSSA and MRSA (Wyche et al. 2012). Some marine bacterium-derived peptides, namely lipoamicoumacins A–D, six known amicoumacins, and a new bacilosarcin C, were extracted from the Red Sea bacteria *Bacillus subtilis*, but only Amicoumacin A showed antibacterial activity versus MRSA (Li et al. 2012) (Fig. 11.2). Also, other Bacillus-derived gageostatins A, B, and C as new linear lipopeptides (containing heptapeptides and new 3-β-hydroxy fatty acids) were isolated from marine bacterium *B. subtilis*. These peptides had potent antifungal activity against pathogenic fungi *R. solani, B. cinerea,* and *C. acutatum* (MICs

values ranged from 4 to 32 μg/mL) and antibacterial activity versus bacteria B. *subtilis, S. aureus, S. typhi,* and *P. aeruginosa* (MIC values ranged from 8 to 64 μg/mL), as well as effective cytotoxicity toward six human cancer cell lines with GI_{50} values of 4.6 to 19.6 μg/mL (Tareq et al. 2014). Kocurin is a new thiazolyl and marine bacterium-derived peptide that separated from bacteria *Kocuria palustris,* and its bioactivity was assessed and the results demonstrated that this peptide is significant inhibitor versus the growth of MRSA MB5393 cell line (Martín et al. 2013). Marine bacteria *Streptomyces scopuliridis* produce four hexapeptides including desotamide and desotamides B-D that are isolated for testing the antibacterial activity, and their inhibitory impact versus methicillin-resistant *S. epidermidis* (MRSE) were positive (Song et al. 2014a, b). Bioactivity of nocardiotide A (a new cyclic hexapeptide) purified from *Nocardiopsis sp.* UR67 strain along with marine sponge *Callyspongia sp.* from the Red Sea against the murine CT26 colon carcinoma, human HeLa cervix carcinoma, and human MM.1S multiple myeloma cell lines was effective, and the results indicated that nocardiotide A as potential marine drug could be applicable compound for treatment the human cancer cells (A. H. Ibrahim et al. 2018). Anticancer evaluating a new lipopeptide as kurahyne B purified from *Okeania* sp. (a marine cyanobacterium sampled from the coast of Jahana, Okinawa, Japan) indicated that this peptide, containing acetylene, inhibits the growth of HeLa and HL60 cells with IC_{50} values of 8.1 and 9.0 μM, respectively (Okamoto et al. 2015). Mathermycin is a new lantibiotic (peptides of family of ribosomally synthesized and posttranslationally modified peptides (RiPPs)) that as an antimicrobial peptide was extracted from *Marinactinospora thermotolerans* SCSIO 00652. Activity assay showed that mathermycin has antimicrobial effect on a *Bacillus* strain (E. Chen et al. 2017). Six tetrapeptides namely actinoramide A, B, D, E, F, and 25-epi-actinoramide A identified from two Streptomyces species, *S. ballenaensis* and *S. bangulaensis,* collected from Costa Rica and Papua New Guinea, respectively, showed potent antimalarial activity (Cheng et al. 2015). Six ilamycin as antimicrobial peptides were purified from deep-sea *Streptomyces atratus* SCSIO ZH16. Activity of these peptides versus Mycobacterium tuberculosis (agent of one of deadliest diseases) was assessed, and peptide E1/E2 exhibited very strong inhibitory activity against the *Mycobacterium tuberculosis* H37Rv growth. Therefore, they can be antituberculosis drug in biomedical treatment (Ma et al. 2017a, b). Two linear polyketide nonribosomal peptides, ariakemicins A and B, were purified from marine bacterium *Rapidithrix sp*. The ariakemicins were comprised of threonine, two ω-amino-(ω-3)-methyl carboxylic acids with diene or triene units, and δ-isovanilloylbutyric acid. Their inhibitory activity on the Gram-positive bacteria growth demonstrated that they can be an antibiotic agent (Oku et al. 2008).

Biological action of grassypeptolide to the process of cancer cell proliferation was examined by Thornburg et al. (2011). For this purpose, extraction of peptides from marine cyanobacterium *Leptolyngbya sp.* was sampled from the *S. Thistlegorm* shipwreck in the Red Sea. Totally, two grassypeptolides D and E were characterized that possess 2-methyl-3-aminobutyric acid and 2-aminobutyric acid and the results of acidity assay demonstrated that every two peptides have strong cytotoxicity against HeLa ($IC_{50} = 335$ and 192 nM, respectively) and mouse neuro-2a blastoma

cells (IC_{50} = 599 and 407 nM, respectively). Lagunamide C separated from marine cyanobacterium *Lyngbya majuscula* shows high-level cytotoxic effect (IC_{50} value ranged from 2.1 to 24.4 nM) on P388, A549, PC3, HCT8, and SK-OV3 cell lines (Tripathi et al. 2010). Two cyclodepsipeptides, veraguamides A and A-G, were purified from cyanobacterium *Oscillatoria margaritifera* and *Symploca cf. hydnoides*, respectively. Every two peptides had high cytotoxic activity, so that veraguamide A of *O. margaritifera* showed potent toxic (LD_{50} value of 141 nM) to H-460 human lung cancer cell line and veraguamides D and E in *S. hydnoides* exhibited notable cytotoxic (IC_{50} value of 0.5 to 1.5 μM) versus HT 29 and HeLa cell line (Mevers et al. 2011; Salvador et al. 2011). Solonamides A and B are another marine peptide that is produced by marine *Photobacterium*. Solonamides A and B had no antibacterial activity against *Vibrio anguillarum* and *S. aureus,* but solonamide B caused reduction in the expression of MRSA hla and rnaIII, which are controlled by agr-dependent quorum sensing system, which can be due to the fatty acid chain of solonamide (Hansen et al. 2018; Machado et al. 2014; Mansson et al. 2011).

11.3.2 Marine Fungi

Marine fungi are one of the most important marine organisms used to produce bioactive natural products including alkaloids, polyketides, terpenoids, peptides steroids, and lactones for drug discovery, which have various activities against bacteria, virus, and cancer cells, and among them, proteins and peptides have low toxicity and side effects for human (Deshmukh et al. 2018; Youssef et al. 2019). Many papers have been published about fungal peptides that we will discuss the biological and pathological properties of a number of peptides extracted from fungi.

Three novel cycloheptapeptide skeleton, cordyheptapeptides C-E (1–3) derived from *Acremonium persicinum,* and peptides 1 and 2 showed cytotoxicity effects on MCF-7, SF-268, and NCI-H460 cancer cells (Chen et al. 2012). Activity of efrapeptins E (4), H (5), F (6), and G (7), linear pentadecapeptides, and N-methylated linear octapeptides (RHM1 (8), RHM2 (9), RHM3 (10), and RHM4) isolated from *Acremonium* sp. versus cancer cells was tested, and the results indicated that efrapeptins E and F have potent cytotoxic activity versus H125 cells, and efrapeptin G has power cytotoxic activity against H125, murine L1210, and HCT-116 cells. Antibacterial activity of RHM1 and efrapeptin G versus Staphylococcus epidermis with MIC was demonstrated (Boot et al. 2006, 2007). From marine fungi, *Aspergillus niger*, cultured in different growth media, three cyclopeptides including cyclo-(l-Trp-l-Ile), cyclo-(l-Trp-l-Phe), and cyclo-(l-Trp-l-Tyr) were isolated (Fig. 11.2), but only cyclo-(l-Trp-l-Tyr) had promoted differentiation of HT-29 cancer cells. However, every three cyclopeptides had antibacterial activity versus *Staphylococcus aureus*, *Escherichia coli,* and antifungal against *A. niger* and *Candida albicans* (Zhang et al. 2010). Hexapeptides namely sclerotide A and sclerotide B were isolated from marine fungus *A. sclerotionum,* and their activity versus fungus *C. albicans* was tested and antifungal effects are reported. Moreover,

sclerotide B showed antibacterial and weak cytotoxic activity against bacteria, Pseudomonas aeruginosa, and HL-60 cell cancer, respectively (Zheng et al. 2009). Similanamide is marine cyclic hexapeptides, which were released from fungus *A. similanensis* coexistence with marine sponge, and its cytotoxic activity was weak in contrast to MCF-7, A373, and NCI-H460 cancer cells (Prompanya et al. 2015). Terrelumamides A and B (lumazine peptides), aspergillamides C and D (isometric-modified tripeptides), and asperterrestide A (cyclic tetrapeptide) were extracted from marine fungus *A. terreus*. By adipogenesis model, terrelumamides A and B exhibited anti-hyperglycemic activity with ascending amelioration of insulin sensitivity in mesenchymal cells of a bone marrow of human (hBM-MSCs). Additionally, asperterrestide A represented effective cytotoxic activity against U937 and MOLT4 human cancer cell lines and inhibitory effects versus an influenza virus of H1N1 and H3N2 strains (Luo et al. 2019; You et al. 2015).

Activity of four cyclic peptides namely psychrophilins E, F, G, and H is isolated from *A. versicolor* evaluated by Oil Red O staining assay, and only type G showed potent effect in reducing lipid (Peng et al. 2014). In other study on A. versicolor, one more new centrosymmetric cyclohexapeptide, aspersymmetide A, and a peptide namely asperphenamate were isolated, which aspersymmetide A exhibited weak cytotoxicity effects versus NCI-H292 and A431 cell lines (Hou et al. 2017). Another peptide derived from *A. versicolor* is cotteslosins A (new cyclopeptide), which showed weak cytotoxicity effects on human melanoma (MM418c5), prostate (DU145), and breast (T47D) cells (Fremlin et al. 2009). Three new peptides namely sclerotiotide L, diketopiperazine dimer A (aspochracin-type cyclic tripeptide), and a cyclic tripeptide were isolated from *A. violaceofuscus*, marine fungus coexistence with sponge, which sclerotiotide L, diketopiperazine dimer A, exhibited anti-inflammatory activity versus IL-10 expression of the (lipopolysaccharide) LPS-induced THP-1 cells (monocytic leukemia cell) (Liu et al. 2018).

Psychrophilin E (cyclic tripeptide) extracted from various marine fungus species of *Aspergillus* sp. especially coexistence with algae showed inhibitory activity against the proliferation of HCT116 (colon) cell line ($IC_{50} = 28.5$ _M) comparable to the standard drug cisplatin ($IC_{50} = 33.4$ _M) (Ebada et al. 2014). Antiviral activity versus herpes simplex virus type 1 (HSV-1) observed in aspergillipeptides D and E was isolated from many *Aspergillus sp.* (Ma et al. 2017a, b). The new cyclic dipeptides (14-hydroxy-cyclopeptine) were derived from *Aspergillus sp.* displaying potent inhibitory versus nitric oxide production in recombinant mouse interferon-activated macrophage-like cell line (Zhou et al. 2016).

Based on the reports of Gulder et al. (2012), lajollamide A (a novel pentapeptide) derived from marine *Asteromyces cruciatus* displayed a weak antimicrobial activity against growth of two bacteria, *Bacillus subtilis* and *Staphylococcus epidermidis*. Two new peptides namely dictyonamides A and B were isolated from marine fungus *Ceratodictyon spongiosum* coexistence with red algae, which type A showed inhibitory activity versus cyclin-dependent kinase 4 (Komatsu et al. 2001). A cyclic heptapeptides, unguisin A and emericellamides B, were isolated from *Emericella Unguis* and *Emericella sp.,* respectively. These peptides exhibited antimicrobial activity against MRSA (methicillin-resistant Staphylococcus aureus strains)

(Oh et al. 2007). *Exserohilum rostratum* is a marine fungus, and four peptides including restrains A, B, C, and D were extracted and that displayed potent cytotoxic activity against (HCT-116) the human colon carcinoma (Tan et al. 2004).

Two new peptides microsporins A and B extracted from a marine fungus, *Microsporum gypseum,* showed histone deacetylase-inhibitory activity versus HDACs and HDAC8 (histone deacetylases), and strong cytotoxic effect against human colon adenocarcinoma (HCT-116). Moreover, microsporin A had effective activity versus the 60 cancer cell panel of the National Cancer Institute (Gu et al. 2007). A new dipeptide, penicimutide, was isolated from *P. purpurogenum* that has potent inhibitory versus HeLa cells (Wang et al. 2016a, b). Bioactivity of some peptides of various marine fungus of *Penicillium* sp. namely gliocladine C, cyclo-(Trp-Ala), cyclo-(Phe-Pro), and cyclo-(Gly-Pro) was tested, and result showed only gliocladine C that inhibits the HepG2 cell growth (Hong and Yang 2011). One marine natural peptide that termed cis-cyclo (leucyl-tyrosyl) was isolated from marine fungus *Penicillium sp* coexistence with sponge that possesses antibacterial effects so that these peptides inhibit the biofilm formation and bacterial growth (Scopel et al. 2013).

Halovirs A, B, C, D, and E (linear peptides) isolated from the marine fungus genus *Scytalidium* effectively inhibit the growth of herpes simplex virus types 1 and 2, so showed antiviral activity. Moreover, halovirs A, B, C, D, and E exhibited activity versus HSV-1 with ED_{50} values of 1.1, 3.5, 2.2, 2.0, and 3.1 μM, respectively (Rowley et al. 2003). A marine fungus namely *Simplicillium obclavatum* contains some peptides such as simplicilliumtides A-M and verlamelins A-B. Simplicilliumtides D showed antifouling activity against the larvae of *Bugula neritina* and bioactivity assay of simplicilliumtides A, E, G, and H demonstrated that these peptides are weakly effective versus human leukemia HL-60 and K562 cell lines, but simplicilliumtide J promotes antifungal effect against *Curvularia australiensis* and Aspergillus versicolor and displayed notable anti-HSV-1 activity (Liang et al. 2016, 2017).

The marine fungus genus *Stachylidium* coexistence with marine sponge has many peptides such as N-methylated peptides (El Maddah et al. 2016). Among these peptides (endolides A, B, C, and D), endolide A showed significant binding effect versus the vasopressin receptor 1A with a Ki value of 7.04 μM, and endolide B had a potent binding effect on serotonin receptor 5HT2b with a Ki value of 0.77 μM (Almeida et al. 2016). Many marine bioactive peptides as peptaibols have been separated from marine fungus genus *Trichoderma* that these peptaibols are cationic and amphiphilic peptides and act as defensive assistant in the innate immune system of many organisms and inhibit versus bacteria and fungi infections (Herbel et al. 2015; Van Bohemen et al. 2016).

Marine peptides isolated from *Zygosporium masonii*, namely zygosporamide as a cyclic depsipeptide, revealed highly cytotoxic activity versus the NCI's 60 cell line panel, CNS cancer cell SF-268, and renal cancer cell line RXF 393 by GI_{50} of 9.1 μM, 6.5, and 5.0 nM, respectively (Torres-García et al. 2014). Talaropeptides A-D were extracted from marine tunicate-derived fungus, *Talaromyces* sp. Activity of all talaropeptides against human lung (NCI-H460) and colon (SW620) carcinoma

cells, the Gram-negative bacteria *Escherichia coli* ATCC 11775 and *Pseudomonas aeruginosa* ATCC 10145, the Gram-positive bacteria *S. aureus* ATCC 25923 and *S. aureus* ATCC 9144, or the fungus *C. albicans* ATCC 10231 was negative, but talaropeptides A and B showed inhibitory effects (IC_{50} 1.5 and 3.7 μM) on Gram-positive bacteria *B. subtilis* ATCC 6633 that are good candidates for pharmaceutical developments (Dewapriya et al. 2018).

11.3.3 Marine Algae

Algae as photosynthetic organism contain chemical compounds such as fibers, minerals vitamins, polyphenols, fatty acids, and proteins and peptides (Álvarez-Viñas et al. 2019; Cardoso et al. 2014), and are important source of secondary metabolites that are interesting for scientists for use in food and human health (Fan et al. 2014; Pimentel et al. 2019). Marine algae have high amount of proteins and peptides that can be useful especially pharmaceutical applications such as antihypertensive, antioxidant, and antidiabetic (Admassu et al. 2018). Most published articles on algal peptides are related to their antihypertensive and antioxidant properties that are isolated via enzymatic hydrolysis of proteins (Chen et al. 2020; Deng et al. 2018; Fitzgerald et al. 2012; Furuta et al. 2016; Lee et al. 2015; Sheih et al. 2010).

Therapeutic activity of a marine peptide derived from red algae *Pyropia yezoensis* named PYP15 versus tested DEX-induced myotube atrophy, and the results suggest that marine algae peptide PYP15 is effective agent in preventing or decreasing the skeletal muscle atrophy (Lee et al. 2019). Two new angiotensin-converting enzyme (ACE)-inhibitory peptides, FQIN [M (O)] CILR and TGAPCR, were isolated and identified from protein hydrolysates of the red algae *Gracilariopsis lemaneiformis* (Rhodophyta) by LC-MS/MS (Fig. 11.2). The activity of these peptides as angiotensin-converting enzyme (ACE)-inhibitory molecules was tested, and the IC_{50} value of those demonstrated that FQIN [M(O)] CILR has better activity against the systolic blood pressure by 34 mmHg (SBP) ($p < 0.05$); so, it was potent ACE-inhibitory peptide (Deng et al. 2018). Also, peptides isolated and hydrolyzed from the protein hydrolysate of the marine macroalga *Ulva intestinalis* via trypsin, pepsin, papain, α-chymotrypsin, and alcalase, named FGMPLDR (Phe-Gly-Met-Pro-Leu-Asp-Arg) and MELVLR (Met-Glu-Leu-Val-Leu-Arg), had angiotensin I-converting enzyme (ACE)-inhibitory activities (Sun et al. 2019a, b). Moreover, ACE-inhibitory activity was observed in peptides including IP (IC_{50} values = 0.020 mg/mL) and AFL (IC_{50} values = 0.023 mg/mL) isolated from *U. rigida* that can be useful for biomedical application as antihypertensive (Paiva et al. 2016).

PPY1 (molecular weight = 532 Da), as a peptide derived by enzymatic hydrolysis from marine red algae *Pyropia yezoensis*, inserted to lipopolysaccharide (LPS) stimulated macrophages of mouse. The result of biological activity analysis showed that peptide PPY1 (K-A-Q-A-D amino acid sequence) inhibited LPS-stimulated nitric oxide (NO) and release of pro-inflammatory cytokines (inducible NO synthase, cyclooxygenase 2, interleukin-1β, and tumor necrosis factor α) and

reduced reactive oxygen species (ROS) that act as anti-inflammatory due to downregulation of extracellular signal-regulated kinase, protein 38, and c jun NH2 terminal kinase phosphorylation in the mitogen-activated protein kinase pathways. Therefore, PPY1 can be an anti-inflammatory drug in the treatment of human diseases (Lee et al. 2015). Two novel peptides with sequences of Gly-Gly-Ser-Lys and Glu-Leu-Ser were identified from proteolytic enzyme hydrolysates of red seaweed laver (*Porphyra* species) for assessing their inhibitory activities against α-amylase enzymes. Property of α-amylase enzyme inhibitory (as agent of control the glucose level of blood) in these two peptides was demonstrated (IC_{50} value of Gly-Gly-Ser-Lys $IC_{50} = 2.58 \pm 0.08$ mM; IC_{50} value of Glu-Leu-Ser $= 2.62 \pm 0.05$ mM). Therefore, these peptides can be used as ingredient or drug to reduce and control diabetes mellitus (Admassu et al. 2018). Through hydrolysis of marine red alga *Gracilariopsis lemaneiformis* proteins (one of ecological and economical source in china) by trypsin, flavourzyme, papain, and alkaline protease, peptide QVEY with amino acid sequence Gln-Val-Glu-Tyr (Mass, 537.57 Da) was identified. Peptide QVEY possessing ACE-inhibitory activity with IC_{50} value of 474.36 μM (0.255 mg/mL) was suggested as agent of reducing hypertension in medicine researches. Identification of prohibiting peptides of hypertension was the goal of Fitzgerald and coworkers, too (Fitzgerald et al. 2012). As for importance of rennin in angiotensinogen system, the first isolation of rennin-inhibitory peptides from macroalgae was done. The red seaweed *Palmaria palmata* protein was separated and hydrolyzed using papain enzyme in order to produce the rennin-inhibitory peptides. Activity of the rennin peptide Ile-Arg-Leu-Ile-Ile-Val-Leu-Met-Pro-Ile-Leu-Met-Ala (IRLIIVLMPILMA) via rennin-inhibitory assay was demonstrated (Fitzgerald et al. 2012). Bioactivity assay of peptides of green microalgae *Chlorella vulgaris* (as a dietary supplement or protein-rich food additive in Japan) was studied by Sheih et al. (2010). They identified a fractioned peptide from algae protein waste via hydrolyzing pepsin, and assessment of its anticancer property demonstrated that hendecapeptide (with amino acid sequence VECYGPN RPQF) has potent inhibitory activity against post-G1 cell cycle arrest in AGS cells, so offered as anticancer compound for more pharmaceutical surveys in future (Sheih et al. 2010). An ACE-inhibitory peptide found in microalgae *Isochrysis zhejiangensis* was identified as FEIHCC (PIZ) and its activity was assessed in human umbilical vein endothelial cells (HUVECs), which showed IC_{50} value of 61.38 μM. This peptide via prevention of enzyme infiltration caused suppression, apoptosis, and reduction in hypertension (J. Chen et al. 2020). Bioactivities of chromopeptides hydrolyzed from C-phycocyanin of blue-green algae *Spirulina sp.* were valued. Five chromopeptides displayed antioxidant and metal-chelating effects and had potent cytotoxic activity against human cervical adenocarcinoma and epithelial colonic cancer cell lines, and they were protected from human erythrocytes toward free radical-induced hemolysis (Minic et al. 2016).

11.3.4 Marine Sponge

Sponges from Porifera phylum are colonial benthic organisms that live in many environments with various ecological conditions from deep sea to shallow waters (Van Soest et al. 2012). Marine sponges along with symbiotic bacteria produce many unique secondary metabolites for survival in such various and harsh conditions that are interested by scientists for human health (Petersen et al. 2020). Up to now, many peptides have been isolated from marine sponges for the usage in the medical researches and drug delivery as anticancer, anti-inflammatory, antimicrobial, and other infection and disorders (Agrawal et al. 2016) that in this chapter a summary of some of the peptides extracted from the marine sponges is described.

Two cyclic peptides, microsclerodermins N and O with a p-ethoxyphenyl moiety, were identified from deep-sea marine sponge *Pachastrella* sp., and these peptides showed cytotoxic effects on HeLa cells. Therefore, they may be helpful in anticancer drug discovery (Fig. 11.2) (Tian et al. 2020). Euryjanicin A discovered from marine sponge *Prosuberites laughlini* is an anticancer cyclic heptapeptide that exhibited high-level activity versus HeLa (human colon) cancer cell line (Anand et al. 2019).

Pharmacological assay of stylissamide G (a bioactive heptacyclopeptide) isolated from marine sponge *Stylissa caribica* from the Caribbean Sea, Bahamas, and purified by the tetrapeptide l-phenylalanyl-l-prolyl-l-phenylalanyl-l-proline methyl ester and the tripeptide Boc-l-leucyl-l-isoleucyl-l-proline, and cyclization of the linear heptapeptide fragment, was done, and the results showed strong anthelmintic effects (antiparasitic) versus worms *Megascolex konkanensis*, *Pontoscolex corethrurus,* and *Eudrilus eugeniae*, as well as high-level antifungal activity toward fungus *Candida albicans* and *Trichophyton mentagrophytes* and *Microsporum audouinii* (Dahiya et al. 2016). MCHs as four marine collagen-derived peptides were hydrolyzed from sponge *Chondrosia reniformis,* and their toxicity, antioxidant, wound healing, and photoprotective activities were evaluated, and the results showed no toxicity against the cell line but incredible antioxidant impact (reactive oxygen species (ROS) scavenging ranging from 23% to 60% for the four MCHs) and protective activity from cell against UV that demonstrate these peptides may be useful in pharmacology as drug and skin protective agent (Pozzolini et al. 2018). 11 peptides including five new callyaerins (I, J, K, L, and M) and six known callyaerins (A, B, C, D, E, F, and G) are identified from the Indonesian sponge *Callyspongia aerizusa*. Based on assay of activity of these peptides against Mycobacterium tuberculosis, THP-1 (human acute monocytic leukemia), and MRC-5 (human fetal lung fibroblast) cell lines, callyaerin A displayed strongest anti-TB activity with MIC90 value = 2 μM (Daletos et al. 2015). Marine sponge *Stelletta clavosa*, sampled from the Torres Strait, contains four new mirabamides E, F, G, and H and one known mirabamide C (five depsipeptides). Inhibitory activities of mirabamide E-F against HIV-1 were notable with IC_{50} values of 121, 62, 68, and 41 nM, respectively (Lu et al. 2011). Also, two anti-HIV peptides were isolated from the marine sponge *Stelletta* sp. (sampled from northwestern Australia) by Shin et al. (2015). In fact, stellettapeptins A and B (as depsipeptides) are the first cyclic nonribosomal peptides that possess a 3-hydroxy-6,8-dimethylnon-4-(Z)-enoic acid

moiety. Peptides A and B were inserted to human T-lymphoblastoid cells infected by HIV-1RF for assessing their actions on HIV. Values of IC_{50} (23 and 27 nM, respectively) demonstrated HIV-1 inhibitory activities of these marine sponge-derived peptides. Seven cyclic peptides callyaerins A-F and H (with 5–9 amino acids and side chains of 2–5 amino acids in length) were cloned from marine sponge *Callyspongia aerizusa*. All peptides showed potent antibacterial activity and exhibited anticancer effects against HeLa and PC12 cancer cells. Callyaerins E and H had effective suppression activities toward the L5178Y cell line (ED_{50} = 0.39 and 0.48 μM, respectively), while callyaerins A displayed potent antifungal impact on *C. albicans* (Ibrahim et al. 2010). A peptide lactone with N-terminus separated from deep-sea sponge *Discodermia sp.* showed cytotoxic activity against HeLa cancer cells (Nakamukai et al. 2018). Activity of marine sponge-derived peptides phakellistatin 17 and 18 against human lung carcinoma cell (A549) and human hepatoma (BEL-7042) was weak, but analogs P18–1 and P18–2 identified by HR-QTOF-MS, 1H NMR, and 13C NMR, especially P18–1, had strong cytotoxic activity against BEL-7042 cancer cells, suggesting anticancer agent production via improving the bioactivity of peptides by reference methods (Wu et al. 2018). ACE-inhibitory peptides present in hydrolysate of marine sponge *Stylotella aurantium* are isolated using hydrolysis enzymes. From many hydrolyzed peptides, the fractions of peptic hydrolysate (<5 KDa) had strongest ACE-inhibitory activity. Two fractioned peptides with amino acid sequences of Tyr-Arg (337.2 Da) and Ile-Arg (287.2 Da) showed ACE-inhibitory activity due to the hydrogen bond interactions and Pi interaction between the dipeptides and ACE. The results demonstrated that peptides of *S. aurantium* can be applicable as antihypertensive drug (Ko et al. 2017). Papuamides E and F (cyclic depsipeptide) were isolated from marine sponge *Melophlus* sp. that had cytotoxic activity (Prasad et al. 2011). A potential activity against fungal infections is observed in theonellamide G that is a marine sponge peptide (a family of bicyclic glycopeptides) derived from the red sea marine sponge *Theonella swinhoei*. Its effective antifungal activity against wild and amphotericin B-resistant strains *C. albicans* with respect to IC_{50} values of 4.49 and 2.0 μM was demonstrated. Additionally, it had cytotoxic effects (IC_{50} = 6.0 μM) on the human colon adenocarcinoma cell line (HCT-16) (Youssef et al. 2014).

11.3.5 Cnidaria

The specific feature of Cnidaria, as oldest multi-organ animals, especially sea anemones (sessile organisms from Anthozoa) and jellyfishes (pelagic animals from Scyphozoan), is nematocysts that are venomous organs for prying and defending themselves against predators (Kayal et al. 2018). These venoms comprise mainly proteins and peptides that are cytolytic or neurotoxic (Schmidt et al. 2019). Peptides of cnidarian venoms and gonads attracted the attention of scientists for use in the medical and pharmaceutical fields (Li et al. 2014a, b) (Andreev et al. 2018) (Zhang et al. 2018). In this section, biological and pharmacological properties of some Cnidaria-derived peptides are mentioned.

Three novel peptides with amino acid sequences of Ile-Gly-Glu-Thr-Gly-Pro, Gly-Ala-Thr-Gly-Pro-Ala-Gly-Tyr-Val, and Gly-Ala-Phe-Gly-Pro-Gly-Gly-Leu-Val-Gly-Arg-Pro were purified from protein of the jellyfish *Rhopilema hispidum* via hydrolysis of protein with response surface methodology (RSM). ACE-inhibitory activities of these peptides (with IC_{50} values 19.07, 27.42, and 31.26 μmol L^{-1}, respectively) demonstrated that those of *R. hispidum* can be considered as antihypertensive compound in clinical trials (Sun et al. 2019a, b). VKP (342 Da) and VKCFR (651 Da) derived from Jellyfish *Rhopilema esculentum* (collected from marine aquaculture farms of Nanjing, Jiangsu Province, China) are two hydrolyzed peptides, which as angiotensin I-converting enzyme (ACE)-inhibitory and antioxidant peptides caused induced activity of the superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-px) in RCMEC cells (Li et al. 2014a, b). One marine peptide with amino acid sequence Ser-Tyr (dipeptide SY) was hydrolyzed by neutral protease from the protein hydrolysates of jellyfish gonad (*R. esculentum Kishinouye*), and valuing the DPPH, _OH, super oxygen anion scavenging activities, and ACE-inhibitory activity indicated that this dipeptide is a potent antioxidant and ACE inhibitor (Zhang et al. 2018). Also, from this species, two collagen peptides CP1 and CP2 were hydrolyzed from proteins of jellyfish by proteinase and papain. CP1 and CP2 with mass <25 kDa had strong effects on the scratch closure in mice due to increasing expression of β-fibroblast growth factor (β-FGF) and the transforming growth factor-β1 (TGF-β1) on collagen peptides. The results indicated that collagen peptides of jellyfish cause increase in the wound-healing process therefore can be a potential drug (Felician et al. 2019). In another study, antioxidant property of peptides of the jellyfish *Rhizostoma pulmo*, Macrì 1778 (Rhizostomae), collected from Mediterranean coastal waters was tested. De Domenico et al. (2019) reported that peptides were hydrolyzed from proteins by pepsin and collagenases. Fractioned peptides showed potent antioxidant activity against stress in human keratinocytes (HEKa) with no cytotoxic effect (De Domenico et al. 2019).

Venom of the sea anemone, *Urticina grebelnyi,* because of acid-sensing ion channel 3 (ASIC3) causes inflammatory and acid-induced pain (Andreev et al. 2018). Two peptides APETx2 and Ugr9–1 were isolated from *Urticina grebelnyi,* and their anti-inflammatory and analgesic activities were tested in comparison with nonpeptide molecules sevanol and diclofenac and for all compounds observed distinct effects on pH-induced ASIC3 current; therefore, these ASIC3 inhibitors are potential drugs for decrease in acidosis-related pain (Fig. 11.2) (Andreev et al. 2018). ASIC-inhibitory activity for peptide APETx2 isolated from sea anemone *Heteractis crispa* was reported, too (Kalina et al. 2018).

Marine sea anemone-derived oligopeptides namely AAP-H and YVPGP were separated from *Anthopleura anjunae,* and their activity against prostate cancer DU-145 cells was assessed. The results showed that these peptides by regulation of the PI3K/AKT/mTOR signaling pathway cause apoptosis via mitochondrial and death receptor pathways and act as antitumor effect versus prostate cancer by induction of cancer DU-145 cell death (Li et al. 2018a, b).

11.3.6 Polychaeta

Polychaeta (bristle worms) is one of the largest classes of annelids; most of them are aquatic and distributed in worldwide (Rouse 2001). Previously, studies on polychaete peptides showed that their natural products can be useful in human health development as therapeutic agents, especially peptides including arenicin-1 that was further studied (Choi and Lee 2012; Orlov et al. 2019; Umnyakova et al. 2018). However, researches on polychaete peptides are only limited to a few study that described below.

Marine antimicrobial peptide, arenicin-1, extracted from coelomocytes of the marine polychaete *Arenicola* was investigated for its activity assay, and the results of tests showed potent biological active versus bacteria, therefore can act as a new anti-infective drug (Fig. 11.2) (Orlov et al. 2019), also, based on testing activity of this peptide on human blood serum, can serve as new therapy agent for the treatment of diseases connected with complement dysregulation (Umnyakova et al. 2018). A novel marine worm-derived peptide namely nicomicin-1 was isolated from skin of marine polychaete *Nicomache minor* (a peptide that combining an amphipathic N-terminal α-helix and C-terminal extended part with a six-residue loop stabilized by a disulfide bridge) and showed high effective antimicrobial activity against Gram-positive bacteria and potent cytotoxicity versus cancer, normal adherent cells, and human erythrocytes (Panteleev et al. 2018). Anticancer activity of marine polychaeta *Perinereis aibuhitensis* peptide (PAP) was valued (Jiang et al. 2019). This peptide as a decapeptide (Ile-Glu-Pro-Gly-Thr-Val-Gly-Met-Met-Phe, IEPGTVGMMF), which was isolated from a marine worm enzymatic hydrolysate, causes stimulation of apoptosis and prevention of proliferation of the H1299 cells, so suggested the PAP as a potential drug for the treatment of human lung cancer H1299 cells, used in clinical researches (Jiang et al. 2019). From marine polychaeta, *echiuroid* worm (*Urechis unicinctus*), one peptide, GELTPESGPDLFVHFLDGNPSYSLYADAVP R with molecular weight of 333 kDa as an anticoagulant compound, was isolated. According to inhibitory assay, this peptide could bind to blood coagulation factor IXa (FIXa) in plasma and inhibited the interaction between FIXa and FX, so suggested that *U. unicinctus* anticoagulant peptide (UAP) has potential in anticoagulant drug production (Jo et al. 2008). A bioactive peptide was found from a marine worm *Eunicidae* sp. (the Asp-Leu-Hse-His-Ala-Gln; mass = 683 Da) that showed antiviral effect against HIV-1 with IC_{50} 3 × 10–5 M (Elyakova et al. 2011).

11.3.7 Mollusca

Mollusca as second largest animal phylum including 70% of animals (identified 52,000 species) produces compounds with medicine applications, especially dolastatin and kahalalide F as anticancer peptides that vastly were used in clinical trials for characterization of their biological effects on human diseases (Benkendorff 2010). Some of the peptides derived from Mollusca with focus on edible gastropods and their biological and pathogenic activities have been explained.

The mantle of marine bivalve, *Mytilus coruscus*, contains high amounts of myticusin-beta (a bioactive 23 amino acid residue peptide) that was isolated from hemocytes and hemolymph and purified by C18 reversed-phase high-performance liquid chromatography. Its antimicrobial activity was tested and showed notable antibacterial effect versus *Bacillus cereus*, *Bacillus subtilis*, *Clostridium perfringens*, *Staphylococcus aureus*, *Streptococcus iniae*, *Streptococcus mutans* (Gram-positive bacteria), *Escherichia coli*, *Pseudomonas aeruginosa*, *Vibrio alginolyticus*, *Klebsiella pneumoniae* (Gram-negative bacteria), and antiparasitic at various concentration, so that could be a antimicrobial and antibiotic in pharmacology and drug industry (Oh et al. 2020). The novel peptide KVEPQDPSEW (AATP) was purified from a marine snail, *Haliotis discus* (abalone, a marine gastropod), and its activity on HT1080 cancer cells was studied. Gong et al. (2019) were reported which AATP has inhibitory effects on MMPs (matrix metalloproteinases that have key role tumor metastasis) and VM (vasculogenic mimicry that causes the promotion of tumor cells) by blocking MAPKs and NF-κB pathways and suppression of AKT/mTOR signaling and suppression of AKT/mTOR signaling, respectively. So, the results displayed effective antitumor activity of AATP peptide that can be suggested for future drug industry (Gong et al. 2019). In another study, Cm-p5 (SRSE-LIVHQRLF) as a antimicrobial peptide cloned from the marine mollusk *Cenchritis muricatus* exhibited antifungal activity versus pathogenic fungus *Candida albicans* (with inhibitory concentration of 10 μg/ml; EC_{50}, 1.146 μg/ml), but had low toxic effects on a mammalian cell line (López-Abarrategui et al. 2015). Marine mollusk-derived bioactive peptides in many studies showed antioxidant activity that described in the following text. Oligopeptide RP1–3-3 with Gly-Asp-Gln-Gln-Lys amino acid sequence and molecular mass of 575.45 kDa was purified from the muscle of short-necked clam (*Ruditapes philippinarum*), and activity assay indicated that this oligopeptide, as an antioxidant agent (that may be because of its Gly residues and lower molecule weight), has DPPH-free radical scavenging activity (IC_{50} = 0.739 mg/mL), reducing power and effective protective activity versus a oxidative DNA damage (Li et al. 2015). Blood clam (*Tegillarca granosa*) contains two peptides BCP-A and BCP-B with amino acid sequences Trp-Pro-Pro and Gln-Pro, respectively, which were purified from its muscle protein hydrolysate (Chi et al. 2015). The results exhibited that BCP-A has potent scavenging activity, as well as high cytotoxicity versus PC-3, DU-145, H-1299, and HeLa cell lines (via scavenging-free radicals and the promotion of death of cancer cells), so as antioxidant and anticancer agent may be useful in drug production (Chi et al. 2015). Other antioxidant peptides such as PIIVYWK (1004.57 Da, P1), TTANIEDRR (1074.54 Da, P2), and FSVVPSPK (860.09 Da, P3) separated from hydrolysates of blue mussel *Mytilus edulis* showed high DPPH radical scavenging and ORAC (oxygen radical absorbance capacity) activity. Additionally, P1 and P3 (having ZnPP, HO-1 inhibitor) had strong potential protective activity toward H2O2-induced hepatic damage (Park et al. 2016). Also, antioxidant activity assay of hydrolysate peptides from edible oysters (*Crassostrea talienwhanensis*) was done (Wang et al. 2014). Two peptides with amino acid sequences proline–valine–methionine–glycine–aspartic acid (PVMGA) and glutamine–histidine–glycine–valine (QHGV)

due to DPPH radical scavenging activities had strong antioxidant effects (Wang et al. 2014). On more peptides namely OPHpap (with 13 amino acids) purified from protein hydrolysate of genus mentioned above, *C. madrasensis* via papain digestion exhibited high antioxidant activity that may be potential drug in pharmacology industry (Asha et al. 2016). From another species of genus *Crassostrea* (e.g., pacific oyster *C. giga*), one new peptide cg Molluscidin (contains lysine–lysine or lysine–arginine amino acid sequence and 5500 Da molecular weight) was derived via ion exchange and C18 reversed-phase high-performance liquid chromatography and its activity against Gram-positive bacteria including *B. subtilis*, *Micrococcus luteus*, and *Staphylococcus aureus* (range of MEC value = 1.3 to 31.3 mg/mL), and Gram-negative bacteria including *E. coli*, *Salmonella enterica*, and *Vibrio parahaemolyticus* (range of MEC value = 0.4 to 2.3 mg/mL) (Seo et al. 2013). Moreover, two antioxidant peptides were observed in the gonad of Scallop *Patinopecten yessoensis*. His-Met-Ser-Tyr (536 Da), a tetrapeptide, and Pro-Glu-Ala-Ser-Tyr (565 Da), a pentapeptide, were identified, and every two peptides exhibited high-level antioxidant activity with IC_{50} values of 3.6 and 16.8 mM hydroxyl radical scavenging activities, respectively (Wu et al. 2016). YSQLENEF DR (Tyr-Ser-Gln-Leu-Glu-Asn-Glu-Phe-Asp-Arg) and YIAEDAER (Tyr-Ile-Ala-Glu-Asp-Ala-Glu-Arg) are two marine peptides, which were derived by a multi-bioassay-guided technique from meat and visceral of sea snail *Neptunea arthritica cumingii* and were identified via liquid chromatography–tandem mass spectrometry. These two peptides, YSQLENEFDR and YIAEDAER, had strong antioxidant, ACE-inhibitory, and antidiabetic effects in zebrafish model via scavenging the reactive oxygen species and protecting from skin cells versus oxidative damage, and because of their low molecular weight, high proportion of hydrophobic and negatively charged amino acids, and specific C-terminal and N-terminal amino acids, may be useful inhibitor of chronic noncommunicable diseases (Zhang et al. 2018). Kim et al. (2012) studied anticancer properties of a peptide isolated from *Mytilus coruscus*. Testing of cytotoxic activity of this peptide (with the sequence Ala-Phe-Asn-Ile-His-Asn-Arg-Asn-Leu-Leu) purified by eight protease for hydrolysis of *M. coruscus* protein demonstrated that it causes death of cells in prostate, breast, and lung cancer cells, and consequently, it can be a marine-derived inhibitor for the prevention of cancer cells. Green-lipped mussel from New Zealand produces the peptide GPH hydrolyzed by pepsin and alcalase enzymes from proteins of mussel. First, this peptide by gel filtration chromatography fractioned to GPH-IV (mass < 5 kDa) showed high-level antioxidant and ACE-inhibitory activities, and second, by reverse-phase HPLC (RP-HPLC) purified to GPH-IV-p2 had strong antioxidant and ACE activities (Jayaprakash and Perera 2020).

Peptides of marine cephalopod had been studied; for example, an antioxidant hexapeptide with /Leu-Asn-Ile/Leu-Cys-Cys-Asn sequence and mass of 679.5 Da isolated from mantle of marine cephalopod *Sepia brevimana* (via hydrolysis of trypsin, α-chymotrypsin, and pepsin) showed notable antioxidant activity (by DPPH (38.81 ± 1.07%) and reducing power assays (0.478 ± 0.03)) and protective effect against DNA damage (because of promotion of the hydroxyl

radical), therefore could be effective as marine natural product in medicine researches (Fig. 11.2) (Sudhakar and Nazeer 2015).

11.3.8 Crustaceans

Crustaceans are one of the most large animal groups that are exposed by various microbes and parasites, and the hemolymph is responsible for its immune system with chemical products for dealing with infections. Proteins and peptides are the main components of these immune systems (Rosa and Barracco 2010).

Peptide, polyphemusin III (with amino acid sequence RRGCFRVCYRGFCFQ RCR) isolated from the horseshoe crab *Limulus polyphemus,* was tested for its antimicrobial and anticancer effects and the findings indicated that this peptide despite antimicrobial activity has cytotoxic effects on human leukemia cell HL-60 via destroying the plasma membrane integrity and increasing cell death, therefore suggested as antimicrobial and anticancer case for further pharmacological studies (Marggraf et al. 2018). A novel antimicrobial peptide namely SpHyastatin isolated from hemocytes of the mud crab *Scylla paramamosain*, with two different proline-rich domain (PRD) and cysteine-rich domain (CRD), indicated different activity against various microbes, so that SpHyastatin had a effect on the surface of *S. aureus* and *Aeromonas hydrophila* whereas it directly killed *Pseudomonas fluorescens* through simultaneous targeting the membrane and the cytoplasm. Moreover, it indicated notable activity on Gram-negative bacteria *Vibrio parahaemolyticus* (Shan et al. 2016). One more peptide as a cationic, amphiphilic α-helical and with the 30 amino acid sequence (MAGGKAGKDSGKAKAKAVSRSARAGLQFPVG RIHRHLK) was identified from the later mentioned crab (*S. paramamosain*) that is named sphistin (Chen et al. 2015). This peptide as a antimicrobial agent exhibited potent activity versus Gram-positive bacteria (*S. aureus*, *C. glutamicum, Bacillus subtilis, Micrococcus lysodeikticus,* and *Micrococcus luteus*) and Gram-negative bacteria (*Shigella flexneri, Pseudomonas stutzeri,* and *Pseudomonas fluorescens*) (Chen et al. 2015). The leg muscle of marine crab, *Charybdis natator*, possess a peptide L-G-L-G-A-A-V-L with molecular weight of 713.456 Da that by trypsin, alcalase, and papain was hydrolyzed, and anti-inflammatory activity was observed due to suppression of LPS-mediated induction of COX-2 in RAW264.7 by leg muscle-derived peptide (Narayanasamy et al. 2020). ACE-inhibitory activity for peptides acquired of shrimp, *Pandalopsis dispar*, via enzymatic hydrolysis of proteins by alcalase and protamex, was tested, and the results showed potent ACE-inhibitory activity (Cheung and Li-Chan 2010). Crustin peptide (Pp-Cru) with molecular weight of 17 kDa was reported from blue swimmer crab, *Portunus pelagicus*. This peptide possesses immunological activities triggering encapsulation, phagocytosis to sepharose beads, and yeast (*Saccharomyces cerevisiae*), respectively. Moreover, antibacterial and antibiofilm activities on *Staphylococcus aureus*, *Enterococcus faecalis* (Gram-positive bacteria) and *Pseudomonas aeruginosa*, and *Escherichia coli* (Gram-negative bacteria) were observed. This antimicrobial property could be applicable in drug discovery (Rekha et al. 2018).

Antilipopolysaccharide factor (AFL) as antimicrobial peptides was found in the shrimp *Rimicaris* sp. habited in the hydrothermal vent of Demos, Manus Basin. Peptides RspALF1 and AFL1P1 were identified from this shrimp that killed many of Gram-negative and Gram-positive bacteria (MIC value ranged from 0.25 μM to 4 μM) and had very high antifungal activity (MIC value = 4 μM to 8 μM). This assay demonstrated antibacterial and antifungal activities of immune system containing AFLs (Fig. 11.2) (Gu et al. 2018).

11.3.9 Echinoderms

Phylum of echinoderms include five classes starfish (Asteroidea), brittle stars (Ophiuroidea), sea urchins and sand dollars (Echinoidea), sea cucumbers (Holothuroidea), and sea lilies (Crinoidea), which are benthic animals (Schillaci et al. 2014). Due to lack of immune system like vertebrate, they adapted to produce many compounds as defensive system against predators, fungal/bacterial/viral/parasitic infections to survive in very harsh and variable (Arizza and Schillaci 2016) that most of these compounds including peptides produce in coelomocytes lysates and celomic fluid and had effective activity against bacterial, fungal, and cancer diseases (Li et al. 2014a, b).

Several peptides isolated from the sea cucumber *Stichopus japonicus* protein by enzymatic hydrolysis are named pepsin peptide (PP), trypsin peptide (TP), papain peptide (PAP), acid protease peptide (AP), and neutral protease peptide (NP). Their antioxidant activities were assessed by hydroxyl radical– (·OH) and superoxide anion (O 2· −)– scavenging activity. TP showed highest activity, and after purification of TP, a subfraction namely TP2b-1 identified with GPEPTGPTGAPQWLR that its radical scavenging activity was IC_{50} = 138.9 and 353.9 μM on OH and on O 2· −, respectively (Zhu et al. 2012).

The results of the ACE-inhibitory activity assay of PNVA and PNLG peptides derived from sea cucumber, *Acaudina molpadioidea* (collected from a local market in Qingdao, China), indicated that these peptides are high potential bioactive compounds in control of hypertension (Li et al. 2018a, b). Mediterranean sea cucumber *Holothuria tubulosa* as a producer of marine natural products contains peptides with antimicrobial properties such as holothuroidin 2 (H2). Activity of new H2 derivative as H2d was evaluated versus strains of the dangerous foodborne pathogen Listeria monocytogenes. H2d showed more effective than H2 against L. monocytogenes. According to the results, this new peptide from sea cucumber may be applicable as antimicrobial agent for suppression of foodborne growth (Cusimano et al. 2019). Activities of two new peptides, strongylocins 1 and 2 (cationic, defensin peptides contain cysteine with mass of 5.6 and 5.8 kDa, respectively) isolated from coelomocyte of green sea urchin, *Strongylocentrotus droebachiensis* (collected from the coast of Tromsø, Norway), against the Gram-negative bacteria *Listonella (Vibrio) anguillarum*, serotype O2 (FT 1801 or AL 104/LFI 6004), E. coli (ATCC 25922), and the Gram-positive bacteria S. aureus (ATCC 9144) and

Corynebacterium glutamicum (ATCC 13032) showed that strongylocins 1 and 2 have strong activities on Gram-negative and Gram-positive bacteria (Li et al. 2008).

The results of antimicrobial activities of three peptides EeCentrocin 1, EeCentrocin 2, and EeStrongylocin 2 purified from the edible sea urchin *Echinus esculentus* (Fig. 11.2) demonstrated that EeCentrocin 1 and EeStrongylocin 2 exhibited effective antimicrobial activity toward the Gram-positive bacteria; *C. glutamicum* and *S. aureus* and against the Gram-negative bacteria, *E. coli,* and *P. aeruginosa* (the ranges of MIC were from 0.1 to 0.78 μM, and 0.7 to 3.13 μM, respectively) (Solstad et al. 2016). The first antimicrobial peptide from sea star was reported by Kim et al. (2018). They discovered peptide PpCrAMP (a cationic peptide rich in cysteine with 38 amino acid residues) from the coelomic epithelium extract sea star *Patiria pectinifera* that showed strong antimicrobial activity.

11.3.10 Ascidians

Ascidians (as sea squirt) are sessile organisms and filter feeders (about 3000 species) that attach to various beds such as rocks, ships and algae, corals, and sponges, and possess a outer covering (tunic) as a exoskeleton with proteins and carbohydrates (Shenkar and Swalla 2011). Ascidians (in particularly, families of Didemnidae, Polyclinidae, and Polycitoridae) associated with symbiotic microorganisms such as fungus and bacteria produce vast secondary metabolites with various therapeutic applications (Chen et al. 2018; Watters 2018). Many discovered peptides from ascidians used in medicine and pharmacology for treating the various diseases, as anticancer (Edler et al. 2002; Won et al. 2015), antimicrobial (Arumugam et al. 2019; Hansen et al. 2020), and antioxidant (Ko et al. 2016), explained below with more details.

A marine bicyclic peptide was separated from two ascidians *Didemnum cuculiferum* and *Polysyncranton lithostrotum*. This 13 amino acid peptide is vitilevuamide that based on its high inhibitory property toward polymerization of purified tubulin had a potent activity versus P388 lymphocytic leukemia of mice, and so could be said that because of cytotoxic activity, vitilevuamide is a possible antitumors in clinical trials (Fig. 11.2) (Edler et al. 2002). Marine ascidian, *Styela plicata,* had an antioxidant peptide with the amino acid sequence ((be Leu-Pro-His-Pro-Ser-Phe (mass of 696.3 Da)) that hydrolyzed nine proteases (Protamex, Alcalase, Neutrase, Kojizyme, Flavourzyme, papain, trypsin, pepsin, and a-chymotrypsin) and identified Q-TOF ESI mass spectroscopy. Based on scavenging, the peroxyl (IC_{50} values of 0.05 mM), hydroxyl (IC_{50} values of 1.98 mM), and DPPH radicals (IC_{50} values of 0.17 mM) and reduction in intracellular reactive oxygen species (ROS) in zebrafish model could be said this peptide has strong antioxidative effects (Ko et al. 2016). Antimicrobial activity assay of a peptide cloned from the Arctic sea squirt *Synoicum turgens* was investigated (I. K. Ø. Hansen et al. 2020). Based on analysis of activity, this peptide (named turgencin A, containing 36 amino acid residues and three disulfide bridges) showed

weak antimicrobial activity, but the optimized peptide StAMP-9 exhibited antibacterial activity toward *S. aureus* and *E. coli*, without cytotoxic impacts on mammalian cells (Hansen et al. 2020). In another study, peptides of ascidians *didemnum* genus (collected from coastal waters of Mandapam, Tamil Nadu, India) for antibacterial activity assay were isolated by buffer assay pathway purified via an RP-HPLC technique. Activity of A peptide P1 (possess seven fractions) recorded (with mass of 40 kDa) against the Gram-negative bacteria, *Enterococcus faecalis* (11.60 mm), at an MIC value of 2.30 μg/ml followed by *Serratia marcescens* (11.26 mm; MIC: 2.17 μg/ml), *Salmonella typhimurium* (10.53 mm: MIC: 2.05 ± 0.01 μg/ml), *S. aureus* (10.43 mm; MIC:1.95 μg/ml), and *Pseudomonas aeruginosa* (10.33 mm MIC: 1.83) was indicated most potent inhibition versus *E. faecalis* and lowest inhibition against *P. aeruginosa*. With regard to the results, peptides of ascidians *Didemnum* sp. can be used as marine drug for therapy of bacterial infection in human (Arumugam et al. 2019). Five peptides separated from ascidians *Ciona intestinalis* as linear cationic α-helical peptides were examined against bacterial infections. All five peptides KH.C1.640, KH.C7.94, KH.S1531.4, KH.S908.1, and KH.S921.1 showed a potent antimicrobial activity toward bacteria *E. coli* and *S. aureus*. Moreover, KH.S908.1 and KH.S921.1 peptides exhibited strong antimicrobial to aerobic bacteria *Pseudomonas aeruginosa*. Also, peptides KH.C1.640, KH.C7.94, KH.S1531.4, and KH.S908.1 had antifungal effects on *Saccharomyces cerevisiae*, demonstrating they may be potential inhibitor for the treatment of microbial infections (Ohtsuka and Inagaki 2020). From ascidian *Aplidium* sp. (sampled from the coast of ChujaDo, Korea), five novel peptides including four dipeptides possess iodobenzene (apliamides A-D) and a dipeptide contains bromotryptophan (apliamide E) were acquired. All peptides showed moderate cytotoxic activity to K562 leukemia cancer cell line ((IC_{50} values of 7.8–21.1 μM), also types B, C, and E had cytotoxicity against A549 lung cancer cell line (IC_{50} 8.3–22.8 μM)), but all showed low activity toward MRC5 human lung fibroblast cancer cell line (IC_{50} > 37.0 μM). Also, apliamide D exhibited notable inhibitory activity versus the enzyme Na^+/K^+ -ATPase, suggesting they are helpful compounds in the development of drug industry (Won et al. 2015). Tunichromes (with one or more dehydrodopa-derived units) isolated from blood of Ascidia nigra showed high activity against the Gram-positive Enterococcus sp. (Sugumaran and Robinson 2012).

11.3.11 Fish

Proteins present in soft fish such as meat, skin, fins, and mucus contain many valuable peptides that hydrolyzed by enzymes (Halim et al. 2016). Bioactive peptides of fishes are potential compounds in drug development for human health that mostly reported as a antihypertensive, antidepressant, antimicrobial, antioxidant, and anticancer (Abuine et al. 2019). The following mentioned some of the fish-derived bioactive peptides along with their activity on some infected human and animal cells.

A series marine bioactive peptides that act as the antioxidant agent were isolated from mackerel muscle protein hydrolysates (MPHs) of *Scomber japonicus* (Pacific chub mackerel) that collected from fish markets of Busan, Republic of South Korea. From these MPHs, 10 peptides were synthesized and antioxidant activity of these peptides was demonstrated, so that peptides ALSTWTLQLGSTSFSASPM and L GTLLFIAIPI exhibited the highest DPPH scavenging (2, 2-diphenyl-1-picrylhydrazyl radical) and SOD-like (superoxide dismutase) activities, respectively (Bashir et al. 2020). In another study, eight peptides with amino acid sequences including Gln-Asn-Asp-Glu-Arg (TJP1), Lys-Ser (TJP2), Lys-Ala (TJP3), Ala-Lys-Gly (TJP4), Thr-Lys-Ala (TJP5), Val-Lys (TJP6), Met-Lys (TJP7), and Ile-Tyr-Gly (TJP8) were purified from the protein hydrolysate of hairtail muscle (HTP). Activity of these eight peptides from hairtail fish (*Trichiurus japonicas*) was analyzed, and the test showed that TJP3, TJP4, and TJP8 have notable scavenging effects on DPPH•, HO•, superoxide anion radical, and 2, 2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical. TJP3, TJP4, and TJP8 exhibited high-level inhibitory effects on lipid peroxidation in a linoleic acid model system. With regard to the acquired results can be said that these peptides especially TJP3, TJP4, and TJP8 have potential for pharmacology applications as antioxidants (Yang et al. 2019). Additionally, they acquired peptides with inhibitory properties against monoamine oxidase A (MAO-A). Peptides hydrolyzed by pepsin and simulated gastrointestinal enzymes (<3 KDa) displayed highest inhibitory activity versus MAO-A ($IC_{50} = 0.61$ and 2.54 mg/mL, respectively). Among 11 peptides identified, peptide VVFEVFW exhibited the most effective MAO-A inhibitory activity. However, all peptides studied may be helpful as antidepressant. A marine bioactive peptide fish as epinecidin-1 (Epi-1) was separated and purified from fish *Epinephelus coioides* (orange-spotted grouper), and its activity was valued versus the protozoan parasite-infected mice and results showed that this peptide has effective antimicrobial activity against *Trichomonas vaginalis*, so could be useful as a antimicrobial peptide (AMP) to produce new drug in therapy of microbial infections (Huang et al. 2018) (Fig. 11.2). Effects of oligopeptide Leu-Ser-Gly-Tyr-Gly-Pro (LSGYGP) purified from tilapia *Oreochromis niloticus* on oxidative stress and endothelial injury in angiotensin II (Ang II)-stimulated human umbilical vein endothelial cells (HUVECs) were assessed and the results demonstrated LSGYP via reduction in oxidative stress and endothelial damage, so it is strong bioactive peptide as antihypertension (Chen et al. 2019). Another peptide from tilapia namely QAGLSPVR isolated from fish skin gelatin hydrolysates had strong antihypertensive effects on angiotensin I-converting enzyme (Sun et al. 2019a, b). The activity valuing of marine fish-derived peptides of hybrid tilapia, *Oreochromis niloticus,* studied with focus on their antibacterial activities and the results of investigation on two peptides, namely tilapia piscidin 3 (TP3) and TP4, showed that the mortality of hybrid tilapia infected with Gram-negative bacteria *Vibrio vulnificus* treated by tilapia piscidin 3 (TP3) and TP4 decreased and therefore could be said these fish peptides may be applicable as marine-derived drug for bacterial infections (Pan et al. 2017). Also, these two peptides had effects on regulation of expression of immune genes (Lin et al. 2016). Additionally, three piscidin ecPis-2, ecPis-3, and ecPis-4

were separated from orange-spotted grouper *Epinephelus coioides*. ecPis-2S, ecPis-3S, and ecPis-4S as the synthetic putative mature peptides had high-level antibacterial and antifungal effects, and ecPis-3S showed strong activity against *Cryptocaryon irritans*, a species of ciliates. Also, assay of the full-length ecPis-2 and ecPis-4 showed that these peptides act as antibacterial, antifungal, and antiparasitic agents. The results indicated that ecPis-2, ecPis-3, and ecPis-4 have key role in immune system of grouper and suggested that ecPis-2S, 2 L, ecPis-3S, and ecPis-4S, ecPis-4 L may be positive applicable in researches related to human health (Zhuang et al. 2017).

Activities of two marine fish peptides, SA-hepcidin1 and SA-hepcidin2, containing cysteine, extracted from *Scatophagus argus* (spotted scat fish) toward Gram-positive and Gram-negative bacteria and versus *Siniperca chuatsi rhabdovirus* (SCRV) and largemouth bass *Micropterus salmoides reovirus* (MsReV) in epithelioma papulosum cyprini (EPC) and grass carp fin (GCF) cells, were examined and the findings demonstrated that two SA-hepcidins have antibacterial activities but only SA-hepcidin2 has antiviral effect and plays important role in immune system of spotted scat (Gui et al. 2016). PaLEAP-2, as marine fish peptide, acquired from *Plecoglossus altivelis,* showed antimicrobial activity against many bacteria (Li et al. 2015). Zebrafish has a peptide that was derived from the C-terminal 55 residues phosvitin. Antibacterial assay of Pt5e versus five clinical multidrug resistance (MDR) bacteria (*E. coli* 577, *E. coli* 4457, *E. coli* 140,237, *Acinetobacter baumannii* 7225, and *Klebsiella pneumoniae* 2182) showed that this peptide killed all MDR bacteria and have strong antibiotic effect and could be a drug agent in future clinical surveys (Li et al. 2016). According to Salger et al. (2016), from the hybrid-striped bass, three class piscidin peptides including the class I piscidins (22 amino acids in length; striped bass and white bass piscidin 1 and piscidin 3), class II piscidins (44–46 amino acids in length; striped bass and white bass piscidin 4; and white bass piscidin 5), and the class III piscidins (55 amino acids in length; striped bass and white bass piscidin 6 and striped bass piscidin 7) were isolated. The results of assay displayed that class I and class II showed antibacterial and antiprotozoal activity and class III peptides had potent antiprotozoal effects (Salger et al. 2016). Two peptides similar to moronecidin were purified from two Antarctic fishes *Notothenia coriiceps* and *Parachaenichthys charcoti* (Shin et al. 2017). The peptide from *Parachaenichthys charcoti* showed antimicrobial activity similar to moronecidin, but peptide of *Notothenia coriiceps* exhibited effective activity more than moronecidin versus bacterium infections and may be more useful in the medical application (Shin et al. 2017). A polyaniline peptide that is named Pa-MAP 1.9 was separated from the polar fish *Pleuronectes americanus*. Activity assay displayed that Pa-MAP 1.9 has effective antimicrobial activity versus Gram-negative planktonic bacteria and biofilms, therefore suggested as potential drug in treatment of bacterial-infected diseases (Cardoso et al. 2016). The synthetic peptide NRC-16 extracted from witch flounder *Glyptocephalus cynoglossus* showed high effective activity against infection of bacteria and formation of biofilm, so can be suggested as antimicrobial and antibiofilm in future health studies (Gopal et al. 2013). The Antarctic icefish *Chionodraco hamatus* contains a new 22 amino acid residue

peptide, the chionodracine (Cnd-m3a), that exhibited strong antimicrobial activity against Gram-human pathogens but low hemolytic and cytotoxic effects toward human primary and tumor cell lines, so it proposed for clinical trials (Buonocore et al. 2019). Using five enzymes including pepsin, trypsin, papain, flavourzyme, and neutrase, two peptides were hydrolyzed from protein of Pacific herring *Clupea pallasii*. Amino acid sequences of these peptides were identified as Leu-His-Asp-Glu-Leu-Thr (molecular weight = 726.35 Da) and Lys-Glu-Glu-Lys-Phe-Glu (molecular weight = 808.40 Da). Activity assessing showed antioxidant effects (IC_{50} values = 1.19 ± 0.05 mg/mL and 1.04 ± 0.06 mg/mL). Based on the results, these peptides can be antioxidant agent in next drug development (Wang et al. 2019a, b).

A marine fish peptide (SCP) was extracted from collagen of skate (*Raja kenojei*) skin. Its activity on lipid metabolism in 17 men (41.2 + 10.4 years old and index of mean body mass = 25.6 ± 1.9 kg/m^2) was assessed. The results indicated that collagen-derived peptide has antiobesity activity against accumulation of lipids and suggested the positive effects of SCP have potential in reduction of overweight of human (Tak et al. 2019). Also, from skin gelatin of thornback ray (*R. clavata*), three hydrolyzed peptides (APGAP, AVGAT, and GIPGAP) were obtained by two proteases. ACE-inhibitory activity of APGAP and GIGAP was reported with IC_{50} value of 170 and 27.9 μM, respectively. However, strongest antioxidant activity was observed via the DPPH radical scavenging assay by AVGAP with a 33% of activity at 3 mg/mL (Lassoued et al. 2015). Also, in one of the newest studies on peptides of seahorse, from SHP-III as the lowest molecular weight fractioned by enzyme of Protamex (SHP) in hydrolyze from seahorse *Hippocampus abdominalis* protein, three peptides with amino acid sequences of Ala-Pro-Thr-Leu, Cys-Asn-Val-Pro-Leu-Ser-Pro, and Pro-Trp-Thr-Pro-Leu were purified. The results of ACE-inhibitory activity assay demonstrated that all peptides of seahorse *H. abdominalis* can reduce the blood pressure of spontaneously hypertensive rat (SHR). Consequently, they may be useful agents for prohibition of act of angiotensin-converting the enzymes (Je et al. 2020). From an anchovy (*Setipinna taty*)-derived peptide with amino acid sequence Tyr-Ala-Leu-Pro-Ala-His, six residues identified that YALPAH, YALRAS, YALPAR, and YALPAG displayed antiproliferative activity against PC3 cells. However, YALRAH had the highest activity (IC_{50} value = 11.1 μM) (Song et al. 2014a, b).

11.3.12 Sea Snake

In traditional cultures like China, chemical products of snake venom have been used in medicine. Natural compounds of venom include peptides, proteins, nucleosides, amino acids, lipids, and carbohydrates that peptides and proteins are the main components of venom and snake uses it as a defensive system and preying (Munawar et al. 2018). With respect to previous studies, peptides and proteins of snake venom have anticancer, anti-inflammatory, antistroke, and analgesic activities (Munawar et al. 2018). One of the favorite peptides of researchers is Cathelicidins,

which are found in many vertebrates (Guo et al. 2017), especially sea snakes (de Barros et al. 2019; Wang et al. 2019a, b; Wei et al. 2015) (Fig. 11.2). These peptides play roles in immune system for fight infections. Hc-CATH is a new cathelicidin that was isolated from sea snake *Hydrophis cyanocinctus*. This peptide with 30 amino acid sequence KFFKRLLKSVRRAVKKFRKKPRLIGLSTLL caused disrupting the bacterial membrane and lysis of bacterial cell, while showed low cytotoxicity to mammalian cell, but had potent antimicrobial activity. Additionally, Hc-CATH is due to inhibition from the LPS-induced production of nitric oxide (NO) and pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6, binding to Toll-like receptor 4 (TLR4/MD2 complex), and activation of LPS-induced inflammatory response pathways showed strong anti-inflammatory effect. The results demonstrated that Hc-CATH as antibiotic can be useful in pharmaceutical researches in future (Wei et al. 2015). From same sea snake species, *H. cyanocinctus*, a peptide, named hydrostatin-TL1 (H-TL), were gained that as anti-inflammatory agent protects from host toward dextran sodium sulfate (DSS)-induced acute colitis and lipopolysaccharide (LPS)-induced acute shock (Wang et al. 2016a, b). Also, hedrostatin-SN1 (H-SN1) extracted from venom gland T7 of *H. cyanocinctus* had potential in treating the TNF-α-associated inflammatory bowel diseases (Zheng et al. 2016).

11.4 Marine Peptides in Clinical Trials and Approved by FDA

Extensive research has been conducted on the production of marine drugs over the past two decades. The first compound used for drug production was cytarabine (marketed name, Cytosar-U®) that derived from Caribbean marine sponge, spongian nucleoside, in 1959 and approved for cancer in 1969, and then vidarabine approved in 1979 as antiviral (Martins et al. 2014). In totally, eight natural product-derived drugs have been approved by Food and Drug Administration (FDA) and/or the European Medical Agency (EMA) that the origin of two drugs is marine peptides (Jaspars et al. 2016; Jimenez et al. 2020). In recent years, increased desire to study biological and pharmaceutical properties of peptides has been observed, so that over 60 peptides are as market drug and many peptides are in clinical pipeline, too (Arumugam et al. 2019). Some of them will be mentioned below, and their mechanism of action in the treatment of pathogens will be explained.

Ziconotide is marine peptide-derived drug with marked name Prialt® approved by FDA in 2004 and EMA in 2005 that purified from venom of cone snail *Conus magus* (Fig. 11.3). In fact, ziconotide is neuroactive peptide that was used to treat chronic refractory pain. Prialt as an analgesic drug (produced by Azur Pharma and distributed worldwide except Europe) blocks N-type calcium channels and inhibited from releasing the neurotransmitter from nociceptive afferents to synaptic space, consequently inhibit the transmission of pain to brain (Deer et al. 2019; Safavi-Hemami et al. 2019) (Fig. 11.3).

Second marked marine peptide-derived drug is brentuximab vedotin (ADCETRIS®) that originated from dolastatin 10, monomethyl auristatin E

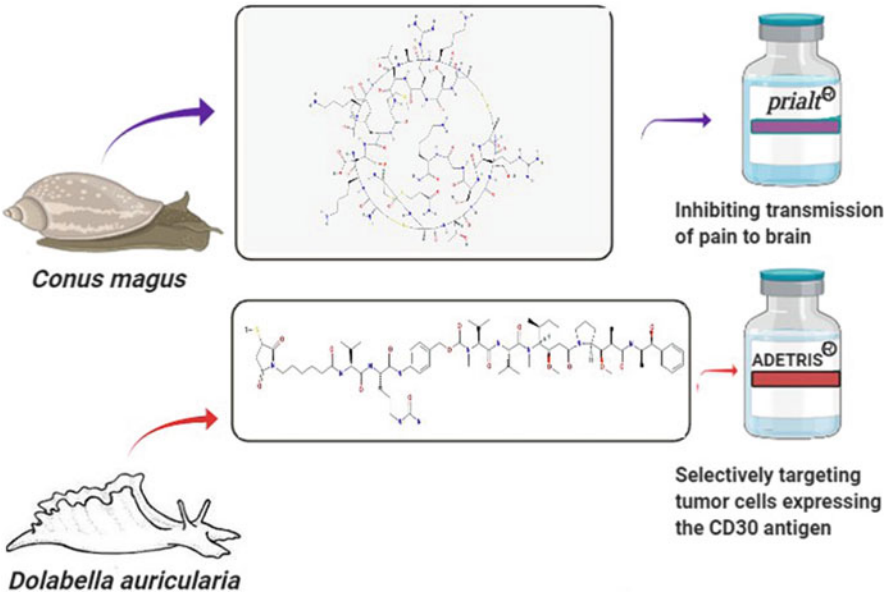

Fig. 11.3 Structures and functions of two peptides derived from marine Mollusca approved as drug by FDA

(MMAE) isolated from the mollusk *Dolabella auricularia*, which inhabit in the Indian Ocean (Fig. 11.3). This drug as antibody–drug conjugate (ADC) was approved by FDA for treating relapsed/refractory Hodgkin lymphoma, systemic anaplastic large cell lymphoma, or primary cutaneous CD30-positive lymphoproliferative disorders (Fig. 11.3). This compound attached to the CD30 antibody, fusion with lysosomes causes introduce MMAE into the intracellular space, binding of MMAE to tubulin disrupts the microtubule network within the cell, resulting induction of G2/M-phase cell cycle arrest and apoptosis (Jimenez et al. 2020).

Glembatumumab vedotin derived from dolastatin 10 is another ADC whose activity as anticancer agent has been studied undergoing phase I/II (Vaklavas and Forero 2014). This peptide by targeting the transmembrane glycoprotein NMB, releasing MMAE into cytoplasm, arresting microtubule, consequently causes cancer cell death (Ott et al. 2019). One of the dolastatin 10 derivatives isolated from marine mollusk *Dolabella auricularia* is soblidotin (phase II clinical trials) that has prohibition activity against murine P388 leukemia, Lewis lung carcinoma, M5076 sarcoma, B16 melanoma, human MX-1 breast cancer, and Colon26 colon cancer cell lines and SBC-3SCLC and LX-1 xenografts (Y. Lee et al. 2017). Cemadotin as analog of dolastatin 15 has antiproliferation and prohibition activity against human tumor and as chemotherapeutic agent of cancer attached to tubulin and stopped mitosis (Jordan et al. 1998). Tasidotin (ILX-651) is another dolastatin 15 that currently is in phase III clinical testing for its pharmaceutical effect assay on solid

tumors, microtubule assembly lung cancer. Tasidotin has antiproliferative effect on MCF7/GFP breast cancer cells ($IC_{50} = 72$ nmol/L), as well as inhibit from polymerization of tubulin (Ray et al. 2007). Another peptide of marine mollusk *D. auricularia* is elisidepsin, analog of kahalalide F (as a marine cyclic peptide) that is in phase I clinical trials because of cytotoxic activity to malignant solid tumors (Serova et al. 2013).

Subfraction HTI-286 of peptide, hemiasterlin (purified from marine sponge *Hemiasterella minor*), has anticancer effects on human cancer cell, especially metastatic prostate cancer due to arresting the mitosis and cell death via inhibition of polymerization and disruption of microtubule (Loganzo et al. 2003). E7974, another analog of hemiasterlin, is in phase II clinical trials for study of colorectal, prostate, and larynx carcinomas. E7974 similar to other anticancer peptides is linked to the tubulin molecule and induce the apoptosis in tumor cells. This compound exhibited potent activity toward colorectal cancer (CRC) xenografts. Also, this peptide has linear pharmacokinetic (PK) properties in mice and dogs and is in preclinical phases for breast cancer, pancreatic cancer, and melanoma (Rocha-Lima et al. 2012).

Two ziconotide derivatives named XEN-2174 (phase II) and leconotide (AM-336, ω-conotoxin CVID; phase I) were isolated from cone snail *C. marmoreus* (Newman and Cragg 2014). XEN-2174, as 13-residue peptide, has inhibitory activity against neuronal norepinephrine transporter. Similar to ziconotide, AM-336 with 27 residue peptide and tree internal CYS-CYS bond blocked calcium channel in cancer cell line (Newman and Cragg 2014). Plitidepsin (trade name aplidin) acquired from ascidian *Aplidium albicans* is in phase I/II clinical pipeline as potential anticancer agent. Actually, plitidepsin is a didemnin compound (a cyclic depsipeptide with ester bonds). This peptide as anticancer is linked to protein Eef1a2 and leading apoptosis in cancers such as lung cancer, melanoma, and multiple myeloma (Alonso-Álvarez et al. 2017; Leisch et al. 2019).

Kahalalide F isolated from mollusk *Elysia rufescens* (sampled from Hawaii), and its diet green algae (*Bryopsis sp.*), is a toxic peptide for defends against predators that were produced by a bacterial or fungal symbiont (Zan et al. 2019). Kahalalide F was first studied by Hamann and Scheuer (1993) as fatty acid–cyclic peptide containing 13 amino acids and 5-methylhexanoic acid at its N-terminus showed strong activity against lung cancer via disrupting the lysosomal membranes, formation of vacuoles, increasing acidification in the intracellular space, and finally induce of apoptosis in cancer cells, and in phase II clinical trials, it had high cytotoxic effect (with IC_{50} values ranging from 0.07 to 0.28 μM) on prostate and breast cancer lines (Rosa et al. 2020; Saadaoui et al. 2020).

11.5 Conclusion

The results of the reviewed papers in this chapter indicated that natural compounds derived from microorganisms including bacteria, fungi, cyanobacteria, algae, fish, and various marine invertebrates contain valuable bioactive peptides as secondary

metabolites that have high potential in the therapeutic and pharmacological areas so that antimicrobial, antifungal, antiparasitic, antiviral, analgesic, antihypertensive, antioxidant, and anticancer properties have been reported for marine peptides. In addition, many marine peptides are under pathological evaluation in different clinical phases for drug production, and two drugs derived from marine peptides, namely ziconotide (Prialtand®) and brentuximab vedotin (ADCETRIS®), were approved by the FDA and distributed worldwide as analgesics and anticancer drugs, respectively. Despite the high potential of marine peptides in the field of health, many marine peptides of marine organisms have not yet been investigated. Therefore, more research is needed to elucidate biological properties of marine bioactive peptides for the development of pharmaceutical industries.

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