Part I
Examples of Natural and Nature-Inspired Materials

Biomimetic Approaches for Biomaterials Development, First Edition. Edited by João F. Mano.

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Biomaterials from Marine-Origin Biopolymers

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1.1
Taking Inspiration from the Sea

Nature has a chemical diversity much broader than chemical synthesis can ever approach. In fact, on the words of Marcel Jaspars, ''*Some chemists, having synthesised a few compounds believe themselves to be better chemists than nature, which, in addition to synthesising compounds too numerous to mention, synthesised those chemists as well.*'' Marine environment is no exception and is being increasingly chosen for the extraction of several compounds, from bioactive molecules to polymers and ceramics. Together with this great potential, one can also find such interesting structures and functions exhibited by diverse marine organisms that biomimetics appears as an extremely attractive approach. Without aiming to be exhaustive, this section presents some examples of those structures and functions and the respective biomimetic approaches.

Biomimetics has been a very attractive route for human scientists and engineers, since the solutions presented by nature to the arising challenges are real engineering wonders, being examples of maximizing functionality with reduced energy and materials. Notoriously, those are precisely the problems faced by the actual engineering challenges to which nature has already given a solution, with the additional advantage of being nonpolluting, in contrast to the majority of the human-engineered solutions [1–3].

For instance, several organisms possess complex and hierarchical structures as a result of their natural growing process, based on self-assembly principles and using relatively few constituent elements [4]. An example can be found in the tree trunk, in which fiber orientation changes with tree growth to optimize the structure and shape of the material as a response to prevailing winds or other environmental constrains, revealing an adaptive mechanical design that may be explored in new engineering solutions. Another example can be found in bone, also revealing adaptive changes to combat external loads or other environmental stimuli. In fact, bone is also an example of a hierarchical biological structure that has been the object of intense research aimed at their mimetization, namely, by tissue engineering approaches [5].

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Nowadays, one of the main purposes of tissue engineering is to produce artificial tissue constructs that possess similar mechanical properties and the capability to trigger specific cellular responses adequate for the tissue to be replaced, mimicking its growth and degradation [6]. In this way, developments in tissue engineering are going toward biomaterials that are competent in biomolecular recognition of tissues adjacent to where they are implanted [7]. Also, the ability of stimulating cellular responses, mimicking extracellular matrix, and guiding new tissue formation are important characteristics that should be considered when creating new biomimetic materials [8]. Such biomaterials and hierarchical structuring, with promising tissue engineering applications, can be found in marine organisms, for instance, in mollusk shells. The mollusk shells are very interesting structures, secreted by the mantle, and composed of different layers. The middle layer is formed by calcium carbonate (calcite or aragonite) and an organic matrix. Also, in some mollusks, this layer is followed by an inner smooth and iridescent surface, the nacre. The nacre is a platelet-tough brick and mortar structure formed by aragonite embedded in a protein matrix [9–11]. Owing to their strong, lightweight hierarchical structure, seashells and, more particularly, nacre have been greatly studied. An independent chapter of this book focuses on the use of nacre-based inspiration to produce high-performance biomaterials. Kamat *et al*. [12] studied shell resistance of the mollusk *Strombus gigas* to catastrophic fracture, concluding that its high resistance is due to the shell lamellar microarchitecture. Asvanund and coauthors studied the osteogenic activity of nacre from the oyster *Pinctada maxima*. In this *in vitro* study, it was shown that nacre induced an increase in the gene expression of osteogenic markers ALP (alkaline phosphatase), BSP (bone sialoprotein), and OC (osteocalcin), demonstrating that nacre is a biomaterial with the ability to stimulate human bone regeneration [13].

Processes of mimicking nacre structure for further use in tissue engineering and regeneration have been disclosed. In 2006, Deville and coauthors unveiled a simple method for the production of a material with lamellar architecture similar to that of nacre. This method is based on the lamellar structure that seawater forms when freezing. In seawater, as freezing temperature is achieved (around $-2\,^{\circ}C$) salts and other particles are excreted and pure water freezes in a lamellar way. In this study, a suspension of hydroxyapatite was frozen, and after being freeze-dried, a layered nacrelike structure was formed. This type of material can be used for bone tissue regeneration [14].

More recently, another method using the layer-by-layer (LbL) technique for producing nacrelike structures has been developed [15]. In this work, the selected polycation was chitosan and the anion was bioactive glass nanoparticles. By this adjustable technique, the authors were able to obtain robust coatings with architecture similar to that of nacre. These coatings can be used in different tissue engineering constructs with applications in orthopedics.

A different marine biomimetic approach can be also envisaged, not based on structural features, but on specific functions evidenced by marine organisms, such as the extremely strong and multisurface adhesion properties of mussels and the variable stiffness exhibited by sea cucumbers and other echinoderms.

Mussels are able to adhere strongly to different wet surfaces by means of their adhesive plaques. In the mussel adhesive proteins (MAPs), the amino acid 3,4-dihydroxyphenylalanine (DOPA) is found in large quantity. It has been hypothesized that DOPA contributes to bioadhesion in sea water [16]. Monahan and Wilker [17] with their study on formation of mussel's adhesives showed that Fe^{3+} enhances the cross-linking ability of mussel adhesives. In brief, iron is extracted from sea water and used in the process of connecting the MAPs together, forming the robust byssus threads. Owing to its strong structure and ability to adhere to wet surfaces, processes of mimicking MAPs for further use in tissue engineering have been developed.

Lee and coauthors [18] synthesized linear and branched DOPA-modified poly(ethylene glycol)s (PEGs-DOPAs) containing one to four DOPA end groups that were able to cross-link into hydrogels when oxidizing reagents were used.

More recently, Burke and coauthors [19] induced the formation of gel of mimicking MAP. An oxidizing agent present in the lipid vesicles with a physiological melting transition of 37 °C is released, and when used in combination with DOPA, rapid cross-linking of the hydrogel was achieved. These stimuli-responsive gels might have the potential to be used for repair of soft tissues.

Podsiadlo *et al.* [20] were able to formulate a nanostructured composite with nacrelike architecture, using DOPA to enhance adhesion and cross-linking. This type of composite might be used for bone tissue regeneration.

Sea cucumbers and sea urchins have the ability to alter stiffness. Their skin is made of collagen fibers embedded in a matrix that can provide low to high stiffness. These collagen fibers are denominated mutable collagenous tissue (MCT). MCTs are similar to mammalian connective tissues in their composition of collagen, proteoglycan, and microfibrils [21].

Muscle is an example of a variable stiffness structure, and the understanding of the mechanism of variable stiffness might be used in ''artificial muscles'' production. Other potential applications have been studied as pharmacological strategies and for composite materials and might be applied when knowledge of the mechanism of MCT mechanical adaptability will be completely elucidated.

A pharmacological strategy that might be used as a therapy for fibrotic lesions in the cervix is the use of holothurian glycosaminoglycans (GAGs). As these GAGs are constituents of a very mutable connective tissue, they might be able to induce the relaxation of the fibrotic tissue when delivered to the lesion [21].

Regarding composite materials, using the knowledge of MCT, different applications for connective tissue replacement and regeneration can be employed. Wilkie [21] gives some examples of some potential applications. One example is the replacement of a complete connective tissue as the Achilles tendon; although a MCT xenograft is not (yet) possible nowadays, some developments have already been done by the study of the echinoid compass depressor ligament and peristomial membrane [22, 23]. Another example is the construction of a composite with constituents extracted from MCT, such as the collagen fibrils. Trotter and coauthors [24] proposed a composite biomaterial that brought together a synthetic interfibrillar matrix with collagen fibrils isolated from holothurians. Also, composite materials

might be created through a biomimetic approach, taking inspiration from sea cucumber's MCT [21].

1.2 Marine-Origin Biopolymers

Marine environment is a source of untold diversity of materials with specific biological and chemical features, some of which are not known in terrestrial organisms. For instance, macroalgae synthesize a great diversity of polysaccharides bearing sulfate groups that find no equivalent in land plants [25] and resemble GAGs found in human extracellular matrix. In spite of this extraordinary potential, the high costs and risks and the lack of technology have hindered a deeper exploration of the marine environment [26]. Nevertheless, in the past decades, new tools and technological developments have allowed to unlock some marine knowledge and in that way, to discover new marine biomaterials to join others already known for many years [26], such as agar [27, 28].

In the present section, the more representative marine biopolymers that find application in the biomedical field are discussed, in particular their chemical nature and the process of isolation from selected marine organisms. Having normally a support function in those organisms, together with other properties, these biopolymers can be considered to be further used in the development of support systems, following a biomimetic approach, for application in tissue engineering scaffolding, discussed later. Moreover, one can see in Figure 1.1 the increasing attention that these biopolymers are receiving from the scientific community, with an increasing number of papers being published in the past 10 years, and these biopolymers are also studied for their correlation with tissue engineering.

1.2.1 Chitosan

Chitosan is composed of D-glucosamine (70–90%) and *N*-acetyl-D-glucosamine (10–30%) units, linked by β(1-4) glycosidic bonds, corresponding to the deacetylated form of chitin, the second most abundant natural polymer, just after cellulose [29]. The difference between chitin and chitosan is determined by the deacetylation degree, that is, the ratio of deacetylated units in the polymer chain, being higher for chitosan, which renders it soluble in dilute acid solutions because of protonation of primary amine groups.

Deacetylation degree is one of the most important characteristics of chitosan, which, together with molecular weight, and also the sequence of repeating units, is responsible for such interesting physicochemical and biological properties possessed by chitosan [29, 30].

Amine groups present in the glucosamine units can be protonated in acidic solutions, as mentioned above, and a way to make it soluble in acid aqueous solutions, which allows its further processability into membranes or gels [31, 32], or

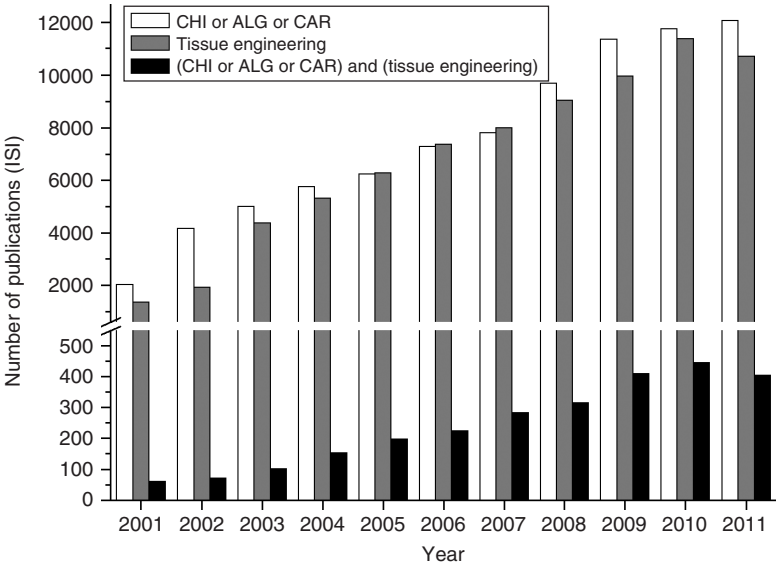

Figure 1.1 Number of published papers in the indicated year, according to the database ISI Web of Knowledge, using the terms "*chitosan* or *alginate* or *carrageenan*" as representation of marine biopolymers, "tissue engineering," and a conjugation of both.

even into particles or fibers, using adequate coagulation solutions (alkali or organic solvents) [33, 34]. Moreover, more complex structures, such as 3D porous structures, can also be obtained by using other techniques, such as freeze-drying [35, 36].

In this charged form, chitosan exhibits a polycation behavior, thus interacting with negatively charged compounds, such as GAGs on the formation of polyelectrolyte complexes [36, 37], or metal complex ions in wastewater treatment systems [38].

Basically, chitin can be isolated from raw materials by the consecutive removal of minerals (by acid treatment, such as 2.5% HCl solution [39]), proteins (by alkaline treatment, such as 2% NaOH solutions [39]), and pigments (by a solid–liquid extraction with acetone or other solvents or a mild oxidizing treatment [40–42]). Acetyl groups are then removed from chitin by treatment with concentrated alkali, thus obtaining chitosan. Two main procedures are commonly referred to in the literature [40, 43, 44] to accomplish this deacetylation: the Broussignac process, in which chitin is treated with a mixture of solid potassium hydroxide (50% w/w) in 1 : 1 96% ethanol and monoethylene glycol, and the Kurita process [45], according to which chitin is treated with hot aqueous sodium hydroxide solution (50% w/v). The reaction time and, in Kurita-based processes, the concentration of alkali and temperature of reaction are parameters that affect the deacetylation degree and concomitantly, degradation of the polysaccharide chain [40].

Commercially, chitosan is mostly produced from chitin derived from marine crustaceans, such as crabs and shrimps [46]. However, it can also be obtained from chitin isolated from other sources, in particular from cephalopods, which has a

different crystallographic form, designated beta, and is characterized by a parallel chain arrangement. This parallel arrangement results in weaker intermolecular hydrogen bonds and consequently more reactive polymers [42]. In this perspective, using β-chitin as a raw material, a more reactive chitosan is produced.

1.2.2 Alginate

Alginate is an unbranched anionic polysaccharide composed of β-D-mannuronic acid (M) and α-L-guluronic acid (G) linked by 1-4 glycosidic bonds [47]. M and G are stereoisomers, differing in the configuration of the carboxyl group, and the position of each unit can also vary, so they can occur in blocks of separate (M or G) or mixed (MG) sequences [48, 49]. The variability in the ratio and sequence of M and G units significantly influences the physicochemical properties of alginate [47, 50, 51].

Alginate is well known for its gelling capacity [47], which is strongly dependent on experimental conditions such as solution viscosity and gelation agent (commonly calcium or other divalent ions) concentration, as well as on molecular characteristics such as molecular weight and structure (M/G ratio and sequence) [52, 53]. In fact, G monomers play a crucial role in the mechanism of ionic gelation, by forming intermolecular ionic bridges induced by the presence of divalent ions, such as calcium [48, 52, 54]. In this way, G-rich alginate will give transparent, stiffer, and more brittle gels, while alginate with higher M content will form more flexible gels [47, 51, 53].

Alginate is the main component of the cell wall of brown algae, thus having not only a structural function but also an important participation in ionic exchange mechanisms [47, 55]. Alginate can be responsible for up to 45% of dry weight of algae, occurring together with other polysaccharides (cellulose, fucoidan, and others) and proteins, with the amount, as well as molecular structure, being dependent on the algal species, environmental conditions, and life cycle [56–59]. For instance, alginates bearing higher G content were extracted mainly from older algae, which thus allow the preparation of stronger gels when compared with materials that are found in younger specimens [59].

The industrial extraction of alginates is mainly done from *Laminaria*, *Macrocystis*, *Ascophyllum*, *Eclonia*, *Lessonia*, *Durvillea*, and *Sargassum* species [60] and starts with treatment with acid solution to convert the magnesium, calcium, and sodium alginate salts found in the cell wall of algae into alginic acid, followed by extraction with sodium hydroxide solution, resulting in soluble sodium alginate. In this way, it is possible to also extract alginates originally complexed with magnesium and calcium ions, which are insoluble, and at the same time to eliminate undesired polysaccharides [61, 62]. The alginate-rich aqueous solution is then separated by filtration, and the alginate can be obtained by precipitation with ethanol, resulting in alginate salt, or by acidification of alginate solution, rendering gelatinous alginic acid [60, 63, 64].

The economically and industrially viable extraction of alginates has been extensively studied, not only to obtain a polymer with controlled and desired properties for a broad range of applications [60], such as gelling agent in food, pharmaceutical, and biomedical industries [65, 66] or as hypocholesterolemic and hypolipidemic agent [67], but also to explore the valorization of by-products, for instance, as source of dietary fibers [62].

1.2.3 Carrageenan

Carrageenans are a family of polysaccharides constituted by 3-β-D-galactopyranose and 4-α-D-galactopyranose repeating units. The structures displayed by this family of polymers allow their organization into three main types according to the number of sulfate groups per disaccharide unit: κ (kappa), ι (iota), and λ (lambda) [68], bearing one, two, and three sulfate groups per disaccharide unit, respectively. In fact, these three types correspond to the commercial names, and the respective polymers are thus obtained after alkaline modification, which involves the conversion of precursor molecules into their final form.

Carrageenans can be found in red algae (Rodophyta), being commonly extracted from the genera *Chondrus* and *Gigartina*. Depending on the life cycle stage, algae can bear different carrageenan types and amounts (between 60 and 80% of dry weight), together with proteins, Floridean starch, and several smaller compounds, some of which exhibit interesting biological activity [69]. Carrageenans can be extracted from red algae by soaking in alkaline bath to perform a chemical modification that enhances the gelling properties, followed by extraction with hot water. Carrageenan can be then recovered by precipitation with salts or organic solvents, such as ethanol [70, 71]. Depending on their use, additional purification steps can be added, in particular, dialysis and reprecipitation, being also wise to firstly remove some contaminants if an extrapure product is desired [72–74].

All types of carrageenans are soluble in water, but at low temperatures, only the λ form is soluble. Of these types, κ and ι carrageenans can be gelified, while the λ form hardly forms gels. Less sulfate content allows the formation of harder gels, but the properties can be further tuned by complexation with other alkali metal ions or by mixture with other polymers [75]. This gelling property makes carrageenans applicable in a broad range of fields, such as in fermentation processes at industrial level [76], as pharmacological excipients [77], or in food products [78, 79] (being designated in the European Union as E407) with stabilizing, thickening, and emulsifying roles [80].

1.2.4 Collagen

Collagen is the most abundant protein in mammals and is formed by three proteic chains that wrap around one another forming a triple helix, with each chain being composed of a specific set of amino acids, namely, repeating triplets of glycine and

two other amino acids, of which proline and hydroxyproline are the most common [81–83].

Collagen has found a wide range of health-related applications, namely, in cosmetics, pharmaceutical capsules, dental composites, skin regeneration, ophthalmology, cardiac surgery, plastic surgery, and orthopedics [84]. Besides, collagen is also used in other sectors, mainly as gelatin, its denaturated form, which is used, for instance, in not only food industry but also photography [85]. Collagen, or gelatin, is the oldest known glue, being used for about 8000 years ago near the Dead Sea [86].

Bovine and porcine bones and skins are the main industrial sources of collagen. However, owing to religious constrains mainly related to Muslim and Jewish customs avoiding porcine products and to active discussion on the risk of bovine spongiform encephalopathy (BSE) and other diseases posed to humans by the use of infected bovine-derived products, other sources of collagen and gelatin are being pursued [87, 88]. Besides recombinant technology using, for instance, the yeast *Pichia pastoris* [89], fish collagen is receiving growing attention. In marine environment, collagen can be found in several marine organisms, with the main sources being marine sponges (e.g., *Chondrosia reniformis*) [90], jellyfish [91–93], and fish bones and skins [81, 94, 95]. Collagen can be obtained from fish skins by treating them with acetic acid solution, and sometimes with the concomitant use of 10% pepsin, thus obtaining acid-soluble collagen and pepsin-soluble collagen, respectively [85]. If jellyfish is used as raw material, it should be washed to desalt and freeze-dried. The material is then treated with 0.5 M acetic acid, and the extracts are dialyzed against 0.02 M Na_2HPO_4. Solid NaCl is then added, and the precipitated fraction is redissolved in 0.5 M acetic acid, dialyzed against 0.1 M acetic acid, and finally freeze-dried [92]. As for collagen derived from fish skins, digestion with pepsin may also be done. When considering marine sponges, the available methodologies are different since sponge collagen is not soluble in acetic acid solution. Thus, Swatschek *et al.* [90] proposed an extraction methodology aimed at scale-up, based on treatment with 100 mM Tris–HCl buffer (pH 9, 10 mM EDTA, 8 M urea, 100 mM 2-mercaptoethanol) for 24 h, with stirring, at room temperature, after which the extract is centrifuged and collagen is precipitated from the supernatant by adjusting the pH to 4 with acetic acid.

1.2.5
Hyaluronic Acid

Hyaluronic acid is a nonsulfated GAG composed of alternating disaccharide units of α-1,4-D-glucuronic acid and β-1,3-*N*-acetyl-D-glucosamine linked by β(1 → 3) bonds [96], with a wide range of molecular weights, which is actually associated with its biological functions. Polymers with molecular weights as high as 10^7 Da have space-filling, antiangiogenic, and immunosuppressive properties, while smaller polymers have anti-inflammatory, immunostimulatory, angiogenic, and antiapoptotic properties [97].

Hyaluronic acid can be found in most connective tissues, such as cartilage, as well as in vitreous humor and synovial fluid [98]. In cartilage, it plays a structural role by

interaction with proteoglycans and proteins [99]. In synovial fluid, hyaluronic acid acts as lubricant and shock absorber, because of its enhanced viscoelastic properties [100]. In fact, difficulty in joint movement and pain as a result of arthritic diseases are due to degradation of hyaluronic acid, which leads to reduction of its viscosity and related properties [101]. Hyaluronic acid also has an important role in the skin, protecting the cells from free radicals that may be generated by UV radiation [102].

Hyaluronic acid can be also found in marine species, in particular in cartilaginous fishes, which can be an alternative source for the production by recombinant technology or by extraction from umbilical cords and rooster combs, first described by Balazs [103] and consists of freezing the materials, thus destroying cell membranes, followed by extraction with water, and then finally precipitating the hyaluronic acid with ethanol, chloroform, or other organic solvents.

1.2.6 Others

Besides the above-mentioned polymers, that together with agar constitute the most representative, studied, and explored marine polymers, other marine polymers are highly promising for biomedical applications, in particular for tissue engineering, and are thus starting to receive growing attention from the scientific community. They are the sulfated polymers chondroitin sulfate (already known, but mainly explored in bovine and porcine cartilages), ulvan, and fucoidan.

Chondroitin sulfate is a polysaccharide that consists of a disaccharide repeating unit of D-galactosamine and D-glucuronic acid, which can exhibit sulfate ester substituents at different positions (the most common are positions C4 or C6 of the galactosamine, corresponding to chondroitin-4-sulfate and chondroitin-6-sulfate, respectively) [104]. Besides an important structural function, chondroitin sulfate also has a role in the development of central nervous system, wound repair, infection assessment, morphogenesis, and cell division [105].

Chondroitin sulfate can be found in the cartilage of several terrestrial species, being explored from bovine, porcine, and chicken cartilages [106, 107], and also in marine species, such as whale [108], shark [109, 110], squid [111], and others [112], being extracted by proteolytic digestion and further purified by precipitation with organic solvents, chromatography, or enzymatic degradation of contaminants [104]. Finally, electrophoretic techniques can be employed for the qualitative and quantitative analyses of chondroitin sulfate [113].

Ulvan represents a family of branched polysaccharides obtained from green algae *Ulva*, with a broad distribution of charge density and molecular weight [114]. It is mainly composed of rhamnose, xylose, glucuronic acid, and sulfate and also contains iduronic acid as a carbohydrate unit [114]. Ulvan is attracting attention as a source of rare sugar precursors for chemical synthesis, but important biological properties are due to its oligomers and polymers, such as antitumor, immune modulation, antiinfluenza, anticoagulant, and antioxidant activities [114].

Ulvan can be extracted from green algae by water extraction, but the purification details are quite relevant for the final use of the polymer. In this perspective,

Alves *et al*. [115] suggested an extraction process starting with extraction with dichloromethane and acetone to remove lipids and pigments, followed by successive extractions with hot water. The liquid resulting from filtration and further centrifugation is submitted to proteinase digestion to eliminate proteins and treated with activated charcoal to remove remaining pigments. Finally, ulvan is precipitated with ethanol and freeze-dried, resulting in a white powder.

Fucoidan is a sulfated polysaccharide existing in two different forms: the major form F-fucoidan mainly composed of L-fucose units, and U-fucoidan, with a significant amount of glucuronic acid units [85]. Several relevant biologic properties have been associated with this polysaccharide, such as antiinflammatory, anticoagulant, antitumoral, and antiviral activities [85, 116]. In particular, fucoidan has been suggested as an anticoagulant as a substituent of heparin [85] and also as an inhibitor of replication of human immunodeficiency virus [116].

Fucoidan can be extracted from some species of brown algae using a hot acid solution and can be further purified by hydrophobic chromatography and dialysis [85]. Ponce *et al*. [116] described a different method of extracting fucoidan from *Adenocystis utricularis* by extraction with 80% ethanol, first at room temperature and then at 70 °C, with the residue being recovered by centrifugation; by treatment with water, 2% $CaCl_2$, or 0.01 M HCl; and finally by dialysis. The use of these different solvents resulted in materials with different properties (sulfate content and monosaccharide proportion) [116].

1.3 Marine-Based Tissue Engineering Approaches

Marine-origin biopolymers have long been proposed for tissue engineering approaches. The natural base character of these materials confers on them adequate properties for applications in tissue engineering and regenerative medicine, mainly because of their low immunogenic potential and chemical/biological versatility and degradability.

Several literature reviews focus on the applications of different polymers, for bone or cartilage tissue engineering. This section specially focuses on the application of marine-based materials. There are several processing techniques available, and the choice of the appropriate one will rely not only on the characteristics of the material itself but also on the final product shape. Depending on each particular application, the material can be prepared in the form of membranes, hydrogels, 3D porous scaffolds, or particles.

1.3.1 Membranes

Membranes have been used in life-saving treatments, such as drug delivery, in diagnostic devices, and as wound dressings, because of its ease in manufacturing and self-application [117]. Solvent casting is a common technique for the preparation

of polymeric membranes. This technique also allows the impregnation of active compounds such as proteins, drugs, or ceramics.

Regarding wound healing, chitosan-based membranes have been shown to be promising candidates [118]. Santos *et al.* [119] have demonstrated, for instance, that chitosan-based membranes do not elicit any inflammatory response, which confirmed the biocompatibility of these types of structures. On the other hand, surface modification of chitosan membranes, particularly by plasma treatment, is another strategy to improve cellular adhesion in matrices for wound healing that has been reported in the literature [120, 121]. An example of a composite porous network for wound healing is the use of bilayered gelatin/chondroitin-6-sulfate/hyaluronic acid membranes [122].

The reinforcement of membranes with ceramic compounds, which can be used for orthopedic applications including in hard tissue regeneration, has been reported by Caridade *et al.* [123] and Li [124].

Recently, LbL processing has gained attention in tissue engineering for the preparation of thin films. The membranes are formed by deposition of alternate layers of a polyanion and a polycation with washing steps in between. Chitosan, owing to its positive charge, is one of the most commonly used natural polycation in LbL processing of nanostructured thin films and was recently reviewed by Pavinatto *et al.* [125]. Marine-origin polyanions include alginates, carrageenans, hyaluronic acid, and chondroitin sulfate.

1.3.2 Hydrogels

Hydrogels are usually defined as water-soluble, cross-linked polymeric matrices containing covalent bonds, hydrogen bonds, physical cross-linkers, strong van der Waals interactions, or crystallite associations [126]. The suitability of hydrogels as biomedical devices is mostly due to their high swelling ability and their soft and rubbery consistency, which resembles most living tissues, and thereafter, natural-based polymers are strong candidates for the development of novel devices. van Vlierberghe and coworkers [127] reviewed the use of natural polymers for the preparation of hydrogels for tissue engineering and regenerative medicine applications. Another review by Oliveira *et al.* [128] gives an overview of the applications of polysaccharides in the development of matrices for tissue engineering. Use in blood vessels, cartilages, corneal stroma, intervertebral disks, meniscus, skin, and tendons is an application of the matrixes developed and reported in the literature. In this section, we focus on hydrogels from polysaccharides and proteins of marine origin.

The feasibility of the use of chitosan hydrogels in these applications has been described in several papers. Its use in different applications implies, however, its modification and/or blending with other polymers (either natural or synthetic) in order to achieve a material with the appropriate characteristics of the particular tissue to be regenerated. Table 1.1 gives an overview of different proposed polymer combinations and their applications.

Table 1.1 Overview of hydrogels from marine-based polymers in different tissue engineering applications.

Polymers	Application	References
Chitosan/hyaluronic acid	Cartilage TE	[129, 130]
Chitosan/alginate	Cartilage TE	[131–133]
Chitosan/gelatin	Cartilage TE	[134–136]
Chitin/nano-hydroxyapatite	Bone TE	[137, 138]
Collagen/chitosan	Blood vessel reconstruction	[139]
Chitosan/gelatin	Blood vessel reconstruction	[140]
Collagen/chitosan	Corneal regeneration	[141]
Alginate	Soft tissues	[142]
Alginate	3D neural cell culture	[143]
Carrageenan	Bone TE	[144, 145]
Carrageenan/fibrin/hyaluronic acid	Cartilage TE	[146]
Hyaluronic acid/fibronectin	Wound healing	[147]
Hyaluronic acid/marine exopolysaccharides	Cartilage TE	[148]
Hyaluronic acid/chitosan or hyaluronic acid/gelatin	Nucleus pulposus regeneration	[149]
Chondroitin sulfate	Cartilage TE	[150]

TE, tissue engineering.

Hydrogels have large applicability, for instance, in cartilage regeneration. Concerning the use of marine-origin-polymers, different hydrogels have been produced based on chitosan/hyaluronic acid [129, 130], chitosan/alginate [131–133], and chitosan/gelatin mixtures [134–136]. Chitin was also processed for the preparation of hydrogels with nanohydroxyapatite particles for bone tissue engineering [137, 138].

In terms of blood vessel reconstruction, some of the most recent works reported include combinations of chitosan/gelatin [139] or collagen/chitosan [140] prepared by freeze-drying.

Collagen/chitosan composite hydrogels have been described as good candidates for corneal treatment. Its preparation as corneal implants involved the stabilization of the matrices with different cross-linking agents [141].

Alginate-based hydrogels have also been prepared for different applications. Park and coauthors [142] evaluated the feasibility of using a novel rapid prototyping technique for the preparation of alginate hydrogels with applications in soft tissues. Alginate hydrogels were also found to be good supporting matrices for 3D neural cell culture [143]. Alginate being a hydrosoluble polymer, cross-linking reactions are required to promote stability of the matrices. Photo-cross-linking methods have now been proposed to create stable systems able to deliver cells without compromising their viability [151].

Carrageenan hydrogels have also been described as potential vehicles for drug delivery in bone tissue engineering [144], and as an injectable system combined with fibrin and hyaluronic acid for cartilage tissue engineering [146]. A comparison

between different carrageenan types and alginate hydrogels as tissue engineering scaffolds was described by Mehrban and coauthors [145].

Hyaluronic acid is another marine-derived polymer that has received great attention, particularly because of its multiple roles in the angiogenic process in the body. Fibronectin—hyaluronic acid composite hydrogels were developed by Seidlits *et al.* [147] in the form of 3D hydrogel networks for wound healing. The continuous growth of the field is demonstrated by the novelty of the materials used in the work of Redderstorff *et al.* [148] in which two new GAG-like marine exopolysaccharides were dispersed in a hyaluronic acid matrix for cartilage tissue engineering. Other applications of hyaluronic-acid-based systems include injectable systems in which hyaluronic acid is combined with chitosan or gelatin for the regeneration of nucleus pulposus [149].

Regarding chondroitin sulfate, which is one of the major components of cartilage extracellular matrix, cross-linked hydrogels were proposed by Wang *et al.* [150] for cartilage tissue regeneration.

1.3.3
Tridimensional Porous Structures

The concept of tissue engineering has long passed the development of an inert matrix and is nowadays based on the development of loaded scaffolds containing bioactive molecules in order to control the cellular function or to act on the surrounding tissues. Hereafter, one of the most important stages of tissue engineering is the design and processing of a porous 3D structure, with high porosity, high interconnectivity between the pores, and uniform distribution so that the cells are able to penetrate the inner part of the network, nutrients and oxygen are accessible to the cells, and cell wastes are eliminated [152]. Conventionally, three-dimensional structures can be obtained by processes such as solvent casting–particle leaching, freeze-drying–particle leaching, thermally induced phase separation, compression molding, injection molding, extrusion, foaming, wet spinning, electrospinning, and supercritical fluid technology, among others [153].

Chitosan scaffolds have been successfully prepared by some of the above-mentioned techniques [154–158]. Figure 1.2 shows as an example a scanning electron microscopic image of a chitosan scaffold produced by supercritical assisted phase inversion.

Together with chitin, these have been by far the most widely studied polymers of marine origin [159, 160]. Chitin scaffolds produced from Verongida sponges were successfully described as matrixes for cartilage tissue regeneration [161, 162]. The difficulties in processing chitin arise due to its high crystallinity and insolubility in most solvents, which prevents its use in a broader range of applications. Alternative green solvents as ionic liquids have been proposed as good candidates for the development of 3D structures with appropriate features for biomedical applications [163, 164].

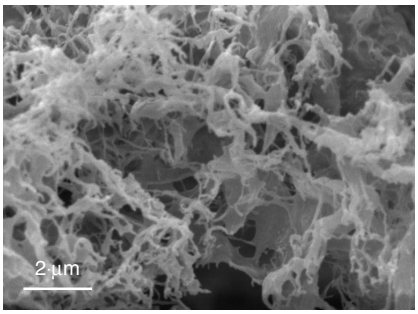

Figure 1.2 Scanning electron microscopic image of chitosan scaffolds prepared by supercritical assisted phase inversion.

Collagen extracted from jellyfish was described by Song *et al.* [92] as a promising novel material for the development of structures with a low immune response for tissue engineering applications.

The use of polysaccharides and/or proteins of marine origin as scaffolds for tissue engineering, namely, for bone tissue engineering is, however, limited because of the poor mechanical properties of these materials and the lack of inherent bioactivity. Nonetheless, this can be easily overcome by the combination of these polymers with other polymers or with bioceramics.

Three-dimensional scaffolds are generally produced to treat bone defects; therefore, special emphasis is given to this application. Polymeric mixtures of chitosan with other marine-derived polymers include combinations of gelatin and chondroitin sulfate for the preparation of 3D matrixes with enhanced properties for bone tissue engineering [165]. To overcome the lack of bioactivity of the polymers and the poor mechanical properties of the ceramics, the preparation of composite matrixes of these two materials is regarded as an interesting approach. Biodegradable composites containing hydroxyapatite-based calcium phosphates (CaP) and Bioglass® can be used to produce promising scaffolds for bone tissue engineering. Inorganic compounds, such as zirconium dioxide and titanium dioxide, are described to enhance osteogenesis, and in this sense, chitin—chitosan scaffolds have been prepared in combination with these compounds for bone tissue engineering purposes [166, 167].

Biomimetic approaches using marine-derived compounds have been reported in different research works, particularly, combining chitosan and calcium phosphates [124, 168–172]. Strategies involving chitosan and hydroxyapatite matrixes have also been described for the treatment of bone defects [173] and osteochondral defects. Oliveira and coworkers [174] describe the preparation of a bilayered scaffold by freeze-drying, while another work by Malafaya *et al.* [33] presents a processing route based on chitosan particle aggregation methodology for the production of cartilage and osteochondral tissue engineering scaffolds.

Ge *et al.* [175] suggest a hydrothermal process to convert calcium carbonate from crab shells in hydroxyapatite and developed a novel composite system of chitosan—hydroxyapatite for bone tissue engineering.

Kusmanto and coworkers [176] describe the conversion of *Phymatolithon calcareum*, a marine alga with a natural interconnected structure of calcium carbonate and hydroxyapatite, while maintaining its structure. Another work reports the production of a 3D matrix from the marine hydrocoral *Millepora dichotoma*, a natural bioactive material that has demonstrated the potential to promote mineralization and differentiation of the mesenchymal stem cells into the osteogenic lineage [177]. The development of hydroxyapatite bone structures, depicted in the work of Cunningham, by replication of three different species of sponges, has also proved to be an interesting approach to the design of novel bioactive structures [178].

1.3.4 Particles

Although 3D porous structures have been recognized as the most appropriate to sustain cell adhesion, several applications in tissue engineering may take advantage of other designs. Injectable systems that can simultaneously act as scaffolds and delivery devices are becoming more and more attractive in this field, especially because of their noninvasive approach [179].

Materials in particulate form for tissue engineering have been reviewed by Silva and coauthors in two papers where the basic concepts and the applications in bone regeneration are described [180, 181]. There is no doubt that tissue engineering strategies are moving toward systems that are able to combine materials, cells, and growth factors. Materials in particulate form can play a role in this strategy as carriers for biologically active molecules. Furthermore, a better control in parameters such as porosity, pore size, surface area, and mechanical properties can be attained in the case of materials in particulate form.

The use of particulate systems for biomedical applications is nonetheless in its embryonic stage of development, and few papers are published in the literature, especially concerning polymers of marine origin.

The applicability of chitosan particles in drug delivery systems has been reviewed by Prabaharan *et al.* [182]. Nonetheless, these particles may also be used for tissue engineering applications, namely, as cell transplantation systems, as described by Cruz *et al.* [183]. These types of particulate systems may also promote growth of bonelike apatite, after a calcium silicate treatment, according to Leonor *et al.* [184]. A technique employed to enhance the mechanical properties of microspheres is to develop multilayer particles, using the LbL technique [185].

Munarin and coworkers [186] described the preparation of calcium alginate, calcium alginate/chitosan, calcium alginate/gelatin, and pectin/chitosan microcapsules for cartilage regeneration.

A combination of chitosan/carrageenan was proposed by Grenha and coworkers [187] as a novel drug delivery device, with potential applications in tissue engineering applications as well.

The development of growth factor delivery systems is one of the most attractive fields of research in particulate engineering. The development of nano- and microscale particles of chondroitin sulfate reported by Lim *et al.* [188] represents a

versatile system for the controlled delivery of positively charged growth factors for a variety of stem-cell-based applications in tissue engineering.

Particulate systems of hyaluronic acid have been developed by Sahiner *et al.* [189] for other types of applications such as vocal fold regeneration.

Marine sponge collagen particles have been developed and were described in two papers on transdermal drug delivery [190, 191]. This is the only work related to the use of sponge collagen in biomedical applications; however, its use can be envisaged in tissue engineering and regenerative medicine.

1.4 Conclusions

Marine life has proved to utilize an immense diversity of ingenious solutions for designing materials. Most of them are true engineering marvels that arose under the same sorts of limitations facing human engineers, such as the need to maximize functionality while minimizing costs in energy and materials. Marine systems are created using nonpolluting processes that occur at biological temperatures and in wet, often salted, environments. Therefore, many researchers have realized that the sea could inspire multiple biomimetic strategies that could be directly applied in the biomedical field. Moreover, the sea has proved to be a huge reservoir of many distinct materials, even though the available knowledge of marine materials and mechanism is still in its infancy. It is envisaged that marine-derived materials will be increasingly explored in biomedicine, in particular in tissue repair and regeneration. Biomedical devices for such applications should be processed into different shapes (fibers, membranes, hydrogels, cellular structures, particles) and sizes. In this chapter, different representative examples were given on how marine-derived biopolymers could be combined and processed into structures for well-defined applications in regenerative medicine.

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