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Biotechnological Valorization of Marine Collagens: Biomaterials for Health Applications

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35.1 Introduction

Collagen is an extremely versatile protein that is almost ubiquitous in the Metazoa; it is the most abundant biopolymer amongst many vertebrates and invertebrates and in mammals it accounts for about 30% of the total protein content [1]. Collagen is a highly hierarchical protein and is the main and more abundant building block of all connective tissues. Close to thirty different collagens have been so far characterized [2]; all of them are composed of three α helices (tropocollagen) that once secreted in the extracellular environment are able to self-assembly into their final supramolecular organization: the paradigmatic left-handed triple helix (Figure 35.1).

Collagens play different key functions in the tissues of adult organisms as well as during their development phases. The primary function of collagen is as a structural component; its ability to resist and dissipate mechanical stress as well as to store elastic strain energy protects the integrity of cells, tissues and organs [3]. Its resistance to strain stress, its great elastic responses as well as the possibility to regulate the supramolecular organization, have been often used by evolution to develop structures with optimized mechanical characteristics, as in the case of tendons in which collagen is arranged to form large aligned collagen bundles, or cartilage and bones where the elasticity and resistance to strain deformation have been combined with the rigidity and compression resistance of the mineral part, or as in the case of cornea where collagen is arranged among different layers with an orthogonal orientation of the fibers to produce an extraordinarily transparent and resistant tissue.

Nevertheless, as anticipated, collagen functions go far beyond from being a simple structural component of the extracellular matrix. Being the main cellular scaffolding molecule, evolution has used collagen as an important player involved in many physiological processes. Indeed, collagen owns specific motifs [1,4], such as the well-known fibronecting-binding RGD motif [5], which are essential for correct development and regeneration of damaged tissues [6–8]. Collagen has thus a critical role in cell fate (adhesion, differentiation) and consecutively in tissue formation and regulation.

The plasticity of collagen molecules in generating different structures is expressed through the different architectures that are often used to classify collagens. The

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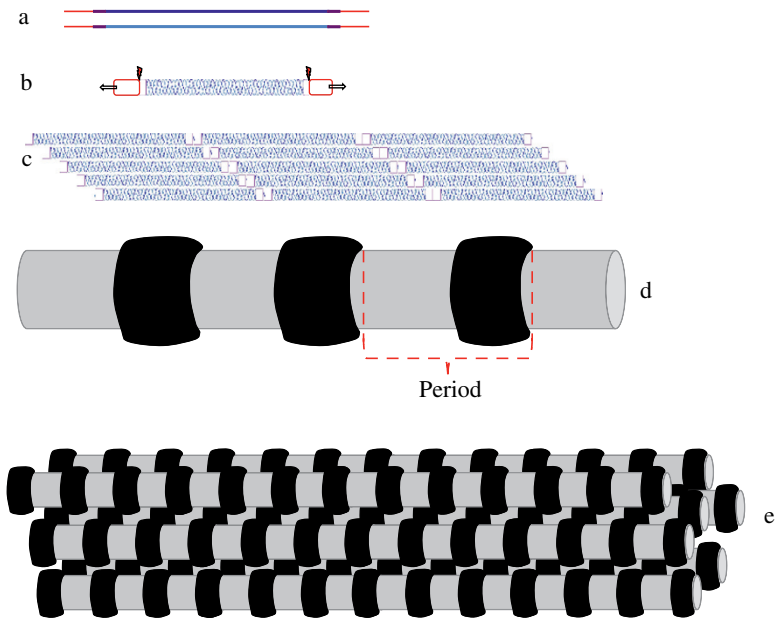

Figure 35.1 Schematic drawing of hierarchical assembling of fibril forming heterotypic type I collagen. (a) The α1 and α2 chains are synthetized; the polypeptides contain a central α-helix part (blue) while at the extremities the telopeptides (purple) precede the N- and C-propeptides. At this stage all the post-transcriptional modifications (as the pro-hydroxylation by the enzyme proline hydroxylase) take place. (b) The N- and C-propeptides help the three chains to correctly assemble forming the α helix; specific enzymes (N- and C-procollagenase) then cleave the N- and C-propeptides. (c) Tropocollagen molecules self-assemble into the typical quarter staggered structure; at this point different interfibrillar bonds are formed in order to stabilize the structure. (d) Collagen fibrils have a typical and regular banding pattern that originates from the presence of gaps between tropocollagen molecules. According to the tissue, collagen fibrils can be organized in different ways: in case they need to form strong and resistant fibers (as in the case of tendons) the fibrils are arranged in parallel fashion. (e) Different interfibrillar crosslinks can be added at this stage.

supramolecolar assembly of collagen helices can form fibrils (anchoring fibrils, microfibrils), transmembrane collagens, networks and basement membranes, among others with unique functions [1,9]. In the present chapter we will focus our attention mainly to the fibril forming collagens type I, II, III; collagen type V and the basement membrane collagen type IV, given either their abundance, relevance to regeneration processes and possibility to purify those collagens from marine organisms (see Table 1 below for specific examples and references).

Traditionally, collagen has been considered as a very promising biopolymer for Tissue Engineering and Regenerative Medicine (TERM), given that collagen is itself the natural cellular scaffolding, is involved in the regeneration of tissues [7], is easy to extract and abundant in nature [10]. Moreover, collagens give very low/no immunological reactions (biocompatibility) and, given the presence of specific degrading enzymes in the human tissues, is biodegradable thus allowing the cells to easily rearrange their microenvironment [11].

Another very well-known and versatile material obtained from collagen is gelatine. Gelatine, which is obtained from the partial and irreversible hydrolysis of collagen, has been used for several applications ranging from the food industry to tissue engineering [12]. Contrary to the native form of collagen, gelatine is characterized by good solubility in water solutions and can reversibly switch from a viscous state to a compact gel depending on the temperature. The gelling process

has been well described and depends on the formation/disruption of hydrogen bonds between amino acids on different polypeptides chains [13].

The last product derived from collagen is represented by collagenous peptides. They can be obtained by the partial hydrolysis and subsequent degradation with different enzymes to obtain specific amino acids sequences, typically exhibiting interesting biological activities [14].

As illustrated by Silva and coworkers [10], marine collagens have gained much attention in the last decade. As a result, much data have been produced, significantly increasing the knowledge of their composition and features allowing comparison of the differences existing between mammalian collagens and marine-derived collagens as well as the differences between different marine organisms.

While the production and use of marine-derived gelatine has been established at the industrial level since the 1980s, more recently collagenous peptides, derived from the enzymatic degradation of gelatine, have entered into the cosmeceutical, pharmaceutical and nutraceutical markets given the demonstrated positive effect on several pathological conditions (as discussed later on). On the other hand, native marine collagens are still very little used in the industrial healthcare field where mammalian sources are still largely dominant [10]. Nevertheless, different factors, such as the contagious risks associated with BSE disease, among others, and the presence of religious constraints concerning the use of bovine and porcine origin materials, have significantly increased the efforts to bring marine collagens from the bench to the markets [10]. Additionally, some marine derived collagens extracted from invertebrates often have peculiar characteristics, described in the following sections, which have yet to be exploited but which are particularly interesting and might shed light on new bioinspired collagen materials.

35.2 Sources of Marine Collagens/Gelatine

Marine environments are much more diverse and rich in species at higher taxonomic levels than terrestrial ones [15,16]. Moreover, marine collagens, in particular those present in the invertebrates, often display singular physiology and properties (e.g. superior or tuneable mechanical properties, resistance to acid treatments, high denaturation temperature, etc.) that might be directly used to produce innovative products or used as a source of inspiration for new functional collagenous materials.

While collagen obtained from terrestrial sources is extracted from few stock species, wild marine organisms, such as fishes and molluscs, are often a valuable and abundant source of collagens (see Table 35.1). Collagen can be also obtained from byproducts of fisheries and fish farms or from bycatch species that have a low commercial value, thus reducing waste production and consequently promoting a better and more sustainable exploitation of natural resources, generating an economic surplus for the fish industrial sector [10].

Fishes are certainly the most investigated source for the extraction of collagen, gelatine and collagenous peptides. Collagen and derived products can be extracted from skin, scales, bones and cartilages. Fish processing can produce from 50 to 80% of solid wastes that contain about 30% of collagen rich tissues currently with generally low to no commercial value [17].

More recently, other marine organisms belonging to the invertebrate group have started to be considered as a diverse source of collagens, either because of their commercial exploitation and resulting production of low value byproducts or for the presence/abundance of specific collagens [18–22].

An overview, as complete as possible, of the collagens successfully extracted from marine organisms, is provided here.

Table 35.1 List of collagens extracted from marine organisms. Collagen type I include homotrimeric and a1a2a3 type I-like collagens.

Species	Group	Ref
Type I		
Aluterus monocerous	Bony fish	[23]
Archosargus probatocephalus	Bony fish	[24]
Evenchelys macrura	Bony fish	[25]
Gadus morhua	Bony fish	[26]
Katsuwonus pelamis	Bony fish	[27]
Lagocephalus gloveri	Bony fish	[28]
Lutjanus vitta	Bony fish	[29]
Merluccius hubbsi	Bony fish	[30]
Parupeneus heptacanthus	Bony fish	[31]
Pleurogrammus azonus	Bony fish	[32]
Pogonia cromis	Bony fish	[33]
Pomadasys kaakan	Bony fish	[34]
Priacanthus tayenus	Bony fish	[35]
Salmo salar	Bony fish	[36]
Sebastes mentella	Bony fish	[37]
Takifugu rubripes	Bony fish	[38]
Theragra chalcogramma	Bony fish	[39]
Thunnus alalunga	Bony fish	[34]
Thunnus albacarens	Bony fish	[40]
Carcharhinus limbatus	Cartilaginous fish	[41]
Chiloscyllium punctatum	Cartilaginous fish	[41]
Scyliorhinus canicula	Cartilaginous fish	[42]
Achanthaster plancii	Echinoderm	[43]
Anthocidaris crassispina	Echinoderm	[44]
Asterias amurensis	Echinoderm	[45]
Holoturia parva	Echinoderm	[46]
Paracentrotus lividus	Echinoderm	[22]
Parastichopus californicus	Echinoderm	[47]
Stichopus monotuberculatus	Echinoderm	[48]
Stichopus japonicus	Echinoderm	[49]
Stichopus vastus	Echinoderm	[50]
Argonauta argo	Mollusc	[51]
Callistoctopus arakawai	Mollusc	[52]
Illex argentinus	Mollusc	[53]

Table 35.1 (Continued)

Species	Group	Ref
Sepia lycidas	Mollusc	[54]
Sepia pharaonis	Mollusc	[55]
Sepia officinalis	Mollusc	[56]
Todarodes pacificus	Mollusc	[57]
Aurelia aurita	Cnidarian	[22]
Cotylorhiza tuberculata	Cnidarian	[22]
Cyanea nozakii	Cnidarian	[58]
Pelagia noctiluca	Cnidarian	[23]
Rhizostoma pulmo	Cnidarian	[23]
Chondrosia reniformis	Poriferan	[59]
Ircina fusca	Poriferan	[60]
Type II		
Carcharhinus limbatus	Cartilaginous fish	[41]
Carcharhinus albimarginatus	Cartilaginous fish	[62]
Chiloscyllium punctatum	Cartilaginous fish	[61]
Rhopilema asamushi	Cnidarian	[18]
Type IV		
Haliotis tuberculata	Mollusc	[21]
Chondrosia reniformis	Poriferan	[63]
Ircina fusca	Poriferan	[60]

Other types/unidentified

Species	Phylum	Type(s)	Ref
Raja kenojei	Chordata	V/XI	[64]
Asthenosoma ijimai	Echinodermata	n.a.	[65]
Dosidicus gigas	Mollusca	n.a.	[66]
Nerita crepidularia	Mollusca	n.a.	[67]
Sepia officinalis	Mollusca	V	[56]
Penaeus indicus	Artropoda	V	[68]
Scylla serrata	Artropoda	V	[19]
Chrysaora quinquecirrha	Cnidaria	n.a.	[69]
Stomolophus nomurai meleagris	Cnidaria	n.a.	[70]
Axinella cannabina	Poriferan	n.a.	[61]
Suberites carnosus	Poriferan	n.a.	[61]

35.3 Extraction Methods for Production of Native Collagens, Gelatine and Collagenous Peptides

Collagen extraction and purification methods, as well as the final yield, mostly depend on the species used as a source, the starting material (i.e. anatomical part) and also the age of the specimens. It is possible to roughly separate collagens into four different categories: acid soluble collagen (ASC), pepsin soluble collagen (PSC), salt soluble collagen (SSC) and insoluble collagen (IC). This division reflects the nature of chemical crosslinks present between collagen fibrils and depends on the factors just identified: the species used as a source, the tissue as well as the age of the specimen.

35.3.1 ASC/PSC Extraction and Purification

The most common treatments used to extract and purify ASC and PSC collagens rely on the much greater stability of native collagen fibers in respect to other proteins. Most of the collagens are barely or not at all affected by alkali treatments and this property is often used to degrade and remove undesired molecules such as non-collagenous proteins. On the other hand, different organic acids, such as acetic acid or lactic acid, are able to solubilize most of collagen macromolecules into their single tropocollagen chains [71]. Although other acids, such as citric acid, have been demonstrated rather effective [72], the most employed on collagen extraction is still a 0.5M acetic acid solution. Pepsin or trypsin enzymes can be added to the acidic medium to solubilize the fraction of collagen (PSC) that has stronger crosslinking bonds at the level of the telopeptide regions; in this case the collagen obtained lacks the telopeptides that are cleaved by the enzyme(s) while the helical region remains intact.

Once the collagen has been solubilized it is generally recovered and further purified through a selective precipitation step using sodium chloride or, less frequently, with K-carrageenan [73]. The pellet can be then resuspended in different acidic medium, dialysed to dilute the precipitating agents and eventually freeze-dried to obtain a sponge-like material.

A more recent approach, based on supercritical fluid technology, where pressurized carbon dioxide is used to acidify water, has been applied to obtain collagen/gelatine from marine species [74]. The method has the advantages to be environmentally friendly, easily scalable, simple and more economic [74]. The main drawback is represented by the quality of the obtained collagen that seems to be less preserved than traditional extraction approaches and resulting in a mix of native collagen and gelatine [74,75]. Nevertheless, its simplicity (no need of pre- and post-processing to remove undesired molecules or to recover collagen/gelatine) might be rather attractive for processing large amounts of material at the industrial level, mainly regarding applications where preserved triple helix is not compulsory [74,75].

35.3.2 SSC Extraction and Purification

Salt soluble collagens are generally a very small fraction in adult specimens [76], but represent a significant portion during the early embryonic development of vertebrates [76]. SSC collagen is significantly more abundant in sponges [60] and echinoderms [77] than in other aquatic groups [78].

35.3.3 IC Extraction and Purification

The term insoluble collagen might be confusing since often the term is used to indicate the fraction of collagen that remains after a standard acidic extraction. In this case most of the remaining fraction can be further solubilized with the enzymatic removal of the telopeptides. We rather refer to IC as collagens that are almost insoluble with acidic treatments also in the presence of enzymes. Those collagens are generally isolated as intact fibrils where the collagen-associated molecules, such as

proteoglycans and glycosaminoglycans, are preserved at the fibrils surface [21].

Those collagens are mainly present in echinoderms and seem to be associated with the presence of peculiar collagenous tissues (MCTs; mutable collagenous tissues) that can change their mechanical states by secreting specific mediators in response to nervous stimuli [79]. Those collagens are characterized by the labile bonds between collagen fibrils that can be isolated directly as fibrils in suspension using solutions containing EDTA and 2-mercaptoethanol [79]. Intriguingly the same solution can be used to extract collagen fibrils from the sponge *Chondrosia reniformis* [80]. Interestingly the *C. reniformis* IC can be also extracted using an alkali treatment followed by an acidic precipitation [81]; while this method has been demonstrated effective with this species, it is still not known if the same method can be applied to isolate MCT's from echinoderms.

Most of the approaches to produce collagen from raw materials generally imply preliminary passages to remove non-desired compound such as salts, minerals, lipids, pigments and non-collagenous proteins. Salts and minerals (such as the ones contained in the bone and cartilages) are generally removed with diluted hydrochloric acid or chelating agents such as EDTA [71]. Lipids are generally removed using organic solvents such as ethanol, butyl alcohol or acetone [71]. Pigments are generally oxidized with hydrogen peroxide and removed with some washes in an alkali (NaOH) solution [82]. As stated above, a further enzymatic digestion might be used to degrade other non-collagenous proteins though the process removes the collagen telopeptides. More gentle processes, based on decellularizing solutions based on SDS, have been also employed to remove undesired proteins and DNA molecules [22] before the collagen extraction steps. All the steps described above are generally performed at lower temperature in order to prevent collagen denaturation and to reduce the activity of possible enzymes that might degrade collagen. It is obvious that marine species that come from cold water, which are more susceptible to thermal denaturation, need to be processed at lower temperatures than tropical species.

35.3.4 Gelatine

Gelatine is generally obtained by thermal treatments of collagen. The pre-treatments used to remove undesired macromolecules are generally similar to those employed for native collagen extraction though more aggressive chemicals and prolonged pre-treatments can be used since there is no need to fully preserve the native conformation of collagen. The production of the water-soluble gelatine from the water-insoluble collagen is achieved by heating collagen in an acidic or alkali medium in order to cleave the intra- and inter-molecular bonds that stabilize the collagen helix. The length of the gelatine polypeptides is one of the most important characteristics of the material and depends on several factors, namely: the species from which the material is obtained; the quality of the starting material; the pre-extraction processes; the time of exposure; the temperature used to denaturate collagen molecules and the pH of the solution [12].

35.3.5 Collagenous Peptides

Collagenous peptides are obtained from the enzymatic degradation of gelatine. Different enzymes (i.e. alcalase, bromein, neutrase, papain, etc.) are used to produce polypeptides chains that are then separated according to the molecular weight and eventually with other chromatographic methods [83]. Here the enzyme and the conditions of the digestions, together with the employed species and tissues, as well as the preservation of the material before the extraction, are the main factors that influence the yield of specific peptides with bioactive characteristics.

35.4 Characterization of Collagens/Gelatine

Although often collagens are mainly characterized in the view of a specific foreseeable use, when possible a complete and standardize characterization, performed in parallel with commercial mammalian collagens, would be preferred given the variety of marine collagen sources and the objective difficulties in comparing different results obtained using slightly different methodologies. The most important aspects that must be assessed when evaluating a purified collagen are related to: its composition; its integrity; the morphology of the fibrils (if a fibrillar collagen is expected); the capability to form a gel and the denaturation temperature. Further investigations are generally performed envisaging specific applications; those generally aim to investigate: the mechanical properties of films/membranes; the porosity of 3D structures; the rheological properties; the biodegradability and biocompatibility *in vitro* and *in vivo*.

35.4.1 Composition

The most rapid and cheap method to gain information on the chain composition and the possible presence of other contaminant compounds is the electrophoresis separation, namely sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE). Samples to be analysed with such techniques can be prepared in mainly two different conditions, namely: in the presence of a disulfide bond reducing agent (DTT or 2-mercaptoethanol) or without it, to evaluate the extent of disulfide bonds. The method is based on the negatively charged SDS and its capability to surround the polypeptide chain of protein, thus giving them a net negative charge. Once a voltage is applied, the proteins migrate through the gel meshwork according to the polypeptide length following the Stroke law. This results in a differential migration speed and in the possibility to resolve the presence of different proteins with rather similar molecular weight (See [84] for a detailed review on SDS-PAGE methods). For collagen generally a polyacrylamide concentration between 4% and 10% is employed and allows the identification of the eventual presence of two different alpha chains as well as the presence of aggregated (beta and gamma) chains. Most of proteins, such as collagen, are easily stained with Coomassie dye thus the same technique can reveal the presence of other contaminant proteins. Other dyes can be used to reveal the presence of other molecules that are less/no sensitive to Coomassie dye [85] or when collagen is highly glycosylated [75]. The exact nature of the alpha chains can be further assessed by a Western blot that is a SDS-PAGE related technique (see for instance [60]. Indeed, once the proteins are separated by the gel electrophoresis the protein can be transferred to an acetate film where a specific collagen chain can be recognized using anti-collagen antibodies. It is important to stress that most of the anti-collagen antibodies have been developed against mammalian telopeptides thus, despite this domain is strongly conserved, they might not always react properly, thus originating false negative results [86]. Collagen extracted using enzymes to cleave the telopeptides are not suitable for characterization using such technique.

To know the exact amino acidic composition of collagen, gelatine or collagenous peptides, the material can be easily processed with automated amino acid analysers. Knowing the hydroxylation level of Pro can give information on the stability of the triple helix and thus its capability to form a firm gel following the acidic neutralization and possibly to resist to higher temperature without being denatured [87]. The extent of Lys hydroxylation can also predict the glycosylation and the stability of the molecule that is also influenced by the ratio of the characteristic Pro and Hyp amino acids (formerly designated, although chemically erroneous, as imino acids) in respect to the total amino acid contents [88].

35.4.2 Integrity of Pro-Collagen Helix

During the extraction process, here comprising the pre-processing steps, many factors can alter or disrupt the collagen triple helix and hence the quality of the extracted molecules. A circular dichroism analysis is generally sufficient to check the integrity of the alpha helix structure [89]. The technique is applied to collagen in solution and is able to assess the structural conformation of the solubilized protein by analysing the absorption of a polarized spectrum of light revealing the presence of alpha helix or beta sheet. Although this technique is the best to investigate the structure of the proteins, being commonly applied to collagen samples to assess the presence of native triple helix, another option is based on another spectroscopy technique, the Fourier Transformed Infra Red (FTIR) spectroscopy. This method, which might also give some hints on the presence of other molecules co-extracted with collagen, has been also used to check the integrity of collagen triple helix by comparing the absorption/reflectance at specific wavelengths [78].

35.4.3 Collagen Refibrillation and Capability to Form a Gel

The process of collagen refibrillation is a spontaneous phenomenon that occurs when an acidic collagen solution is neutralized. The refibrillation process, which depends on the ionic composition of the medium, the temperature and pH [90,91], can be easily observed using a spectrophotometer performing a turbidimetry assay [90,92]. Collagen can be refibrillated alone or in the presence of other molecules such as proteoglycans or glycans that often affect the final morphology of the fibrils obtained [93]. The aspect of the fibrils/fibers is generally observed using a transmission electron microscope [94] or an atomic force microscope [59,93].

In fact, different electron microscopy techniques are generally used to investigate the morphology of collagen fibrils or collagenous structures, in native tissues or in isolated materials after refibrillation. The analysis of collagen fibrils aims to acquire information on their overall morphology, diameter, length, aspect ratio (diameter/length), homogeneity, presence and periodicity of the banding pattern, as well as the possible presence of collagen associated molecules such as proteoglycans [22,59,94].

Some collagen solutions, above a specific concentration threshold, have the capability to form a firm gel following the neutralization of the pH [87]. The possibility to obtain a firm gel from a collagen solution largely increases its possible uses, namely as a direct strategy to produce hydrogels, attractive to several pharmaceutical and biomedical applications.

35.4.4 Denaturation Temperature

The denaturation temperatures of collagen molecules derived from marine sources are generally lower than those obtained from terrestrial sources since the former have less Hyp content. This has been associated, from an ecological point of view, to the temperature of the living environment of those species. The method of choice for investigate the transition from a native collagen triple helix to a denatured form is the differential scanning calorimetry (DSC). The machine heats the sample and analyse the heat flow; during the denaturation phase the sample absorb energy while once the molecules are denatured the energy (heat) absorbed by the sample is directly transformed into Brownian movements. The presence of other peaks in the thermogram can indicate the presence of other molecules, including individual collagenous polypeptide chains experiencing gelation, but the method is generally not very sensitive to small contaminant proteins and other methods are preferred to check the purity of the batch (see paragraph 35.4.1).

While circular dichroism has been rarely used to investigate the effects of temperature

on the stability of collagen [95], probably due to the time consuming and complex methodology when compared to others, another popular approach to measure collagen denaturation temperature is rheology. This approach is based on the rapid change in the viscosity observed when collagen is denatured. The denaturation temperature is calculated as the temperature at which 50% of the fractional viscosity is lost [68,78].

It is important to highlight that the denaturation temperature of collagen is significantly affected by both its intrinsic properties as well as the experimental setting/method used to calculate it. In specific, while the intrinsic denaturation temperature of each source depends on its amino acid composition and the level of Hyp in particular, it has been demonstrated that the denaturation temperature is higher when faster heating rate is applied to collagen [96], reflecting the influence of process kinetics. Moreover, denaturation temperature is more appropriately measured with samples in aqueous solutions or, eventually, gels, rather than solids, to give molecules the adequate mobility and thus more accurately evaluate the process, avoiding, for instance, the influence of moisture on the results.

35.4.5 Rheology

When collagen, alone or in combination with other molecules, must be processed or employed as a drug or cell carrier for local applications or injected for tissue augmentation, it is essential to proper investigate its properties and behavior under flow conditions [97]. The proper tool to investigate the response of polymers (or biopolymers as in this case) is rheology. Rheological measurements investigating collagenous material's responses under dynamic conditions allow to assess, and eventually adjust, the composition of the formulations in order to achieve the desired characteristics [97,98].

So far, rheological measurements have been rarely carried out on native collagens while they have been extensively employed on gelatine (see [12] for and extensive review). Indeed rheology of native collagen has been mainly used to assess the denaturation point of native collagen solutions [50,54], as mentioned above, rather than to investigate the behavior of collagen under flow conditions towards an envisaged application. Rheology can be also particularly useful to study the tropocollagen assembling process and the sol–gel transition [99].

While the intrinsic properties of mammals type I collagen solutions (alone or in combination with other materials, after being chemically modified and under different ionic or pH conditions) have been investigated by several authors [97,99–102], less is known about marine-derived collagens. Moreover, marine collagens potentially rely on a very large set of different sources, thus making direct comparisons between mammalian and marine-derived collagens rheology using literature data a challenging task, also because the experimental settings of rheological experiments have a broad range of possibilities. Nevertheless, one can postulate that overall marine collagens are characterized by a performance similar to the mammalian collagen shear thinning behavior [103,104]. Comparative studies performed in parallel with mammalian collagen showed that both ASC and PSC from shark skin have lower storage and loss modulus when compared to ASC and PSC extracted from pig skin, with higher tan ∂ values [103]. By its turn, solutions of catfish *Mystus macropterus* collagens were found to have lower modulus and complex viscosity and higher tan ∂ values, bringing to the conclusions that the entanglement effect was weaker in fish collagen solutions [104].

Fish derived gelatine properties, deeply reviewed by Karim and Bhat (2009) [12], strongly depend on: the habitat (i.e. temperature, which is also often related to Pro/Hyp content) and the physiology (i.e. tuna species have high body temperature in respect to their water environment; [105] of the different species; the extraction conditions [106,107] and

the molecular weight composition [12,108]. Fish-derived gelatines are rather heterogeneous in their properties but, in general, they are higher in viscosity while have lower melting/gelling point [12]. The capability to form gels is also lower for cold fish-derived gelatine while it is worth to remark that fish gelatine gels continue to increase their strength after the gelation, potentially becoming significantly stiffer than mammalian gelatine [12].

35.5 Processing and Crosslinking of Collagen

Native collagens can be used, alone or in combination with other materials, to produce membranes, particles, matrices, hydrogels and porous scaffolds. Membranes are generally produced by solvent casting techniques but might also be obtained using more complex methods to produce aligned fibers. Collagen alignment is more often achieved using the electrospinning technique that is relatively simple and effective [109]; nonetheless other more complex methods have been proposed [101,110–111]. Collagenous hydrogels can be obtained by neutralizing acidic collagen solution using concentrated strong bases (in order not to dilute the collagen solution). The gel is the result of the collagen reassembling process, which is affected by the temperature [91, the ionic environment [91,112] and the presence of other molecules [91, 93]. So far few (marine) collagens have shown the capability to directly produce hydrogels following the neutralization step. On the other side, porous scaffolds can be obtained by freeze-drying collagen solutions. The final architecture mostly depends on the collagen concentration and freezing rate (i.e. slower the temperature, more time is given for ice crystals to evolve and consecutively bigger pores result after sublimation [113]. Another innovative and potentially disruptive method to process collagen is the technology of 3D printing; this aspect will be treated below, in a separate section.

Independently from the structure obtained and the methods used to produce it, a further step to stabilize collagen and increase its mechanical properties and chemical stability (i.e. denaturation temperature and degradability) is generally needed; this step consist in the introduction bonds between different collagen fibrils and is generally referred as collagen crosslinking. Stabilization of collagen can be achieved using physical, chemical or enzymatic techniques. Physical methods have the advantage that they avoid any chemicals or residues that might be cytotoxic or poorly biocompatible; the most used are dehydrothermal treatments, UV or gamma irradiation. Chemical crosslinks are in general stronger that physical methods [114]. So far the most used crosslinking agents are 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC)/N-hydroxysuccinimide (NHS), given its strong crosslinking capabilities and the lack of potential toxic residues, contrary to the case of glutaraldehyde-based crosslink. Besides, genipin is also being explored as chemical crosslinking [42], claiming a more biocompatible behavior given its natural origin, while hexamethylene diisocyanate (HMDI) has been used in the medical devices industry to crosslink porcine collagen, namely on Permacol™ surgical implant (from Covidien, now Medtronic), and currently under research to crosslink marine collagens in our labs. Enzymatic crosslink are relatively new and have been less used to stabilize marine collagens despite their good potentialities. In particular, transglutaminase [115] is thought to give collagen a more natural crosslink.

Each approach has its advantages and drawbacks and the choice is often driven by the requested mechanical properties of the construct, the presence of living cells, the dimension and architecture of the construct as well as the final potential uses of the produced materials that must be economically competitive with similar products. Nevertheless it is important to highlight that while strong collagen crosslink might work well in *in vitro* studies, the situation

can be reversed after the collagenous materials have been implanted [116].

35.5.1 Specific Characterizations Envisaging Biomedical Applications

Besides the collagen intrinsic properties addressed in previous sections of this chapter, other important characteristics that influence the success of a given collagenous structure, like the ones just described, as a biomedical implant are related to specific properties of the constructs. We will here briefly discuss some of the most common features that are important to assess.

35.5.1.1 Mechanical Investigations

The mechanical properties, which can be partially tailored namely through crosslinking procedures, are obviously important when collagenous structures must resist the mechanical stress after being grafted, but also when collagenous structures are laden with cells or stem cells. It is indeed well known that mechanical properties of a specific substrate have significant influence on cell physiology as well as on stem cells differentiation [117–119]. The mechanical properties are often assessed in dynamic conditions using a dynamic mechanical analyser [120], while in static conditions different models of force transducers are employed [22].

35.5.1.2 Porosity of 3D Structures

The presence of empty and intercommunicated pores/canals inside a collagenous 3D porous scaffold or hydrogels, as well as the mesh size of collagenous membranes, are important aspects that can promote/inhibit the movement and migration of cells or block/retard the penetration of pathogens [121]. While the mesh size of membranes, which are generally rather thin, can be evaluated using a SEM, for more complex and thick structure the knowledge of the inner architecture is achieved more precisely using micro computer-assisted tomography (μCT) [122] or focus ion beam scanning electron microscopy (FIB-SEM) [123]. Through μCT images it is then possible to calculate different parameters (empty space to address mean pore size and porosity, pores interconnectivity, dimensions of inner structures) directly using the software tools and appropriate mathematical models. When very accurate measurements are needed traditional histological or ultrastructure investigations might be employed, but they require significant experience to understand the overall architecture and are significantly more time-consuming than automatized instruments such as μCT.

35.5.1.3 Biodegradability

Ideally, medical devices aiming to promote or accelerate the full regeneration of damaged tissues must be replaced by new, fully functional, tissue over the time. Thus, the materials used must be sufficiently stable to assist and promote the regeneration process while, at the same time, being sensitive to specific enzymes secreted by cells and used to rearrange the extra cellular matrix during the regenerative steps (biodegradability). Although *in vitro* it is not possible to fully mimic the exact conditions of an implanted material, specific and standardized experiments can give hints on the response of the material when exposed to different enzymes. As far as collagenous material is concerned most of the *in vitro* assays evaluate the degradation of a collagenous structure by their dry weight loss upon incubation in aqueous medium in the presence of collagenase [124].

35.5.1.4 Cytocompatibility/Biocompatibility

A material is defined as biocompatible when the reactions following the contact of the material with cells or tissues are below a specific threshold. Those experiments are fundamental to demonstrate the medical potentialities of a material and have been standardized under the ISO 10993 guidelines in order to make easier to evaluate and compare the performances of different materials as well as to guarantee the safety of new materials.

To be biocompatible a material must not trigger a severe inflammation/immunity reaction once implanted. Nevertheless, a small and controlled inflammation reaction is not considered fully negative since it has been demonstrated that it accelerates the healing/regeneration process [125].

35.6 Current Applications

While marine collagens have showed to be a suitable material for different biotechnological applications [10], so far no products have yet reached the biomedical market, particularly if considering end-user applications (a few testing products start to arise, but only in business-to-business situation). If this might partly be justified by the attention given to alternative collagen sources being rather recent, it is also worth stressing that other constrains, such as the availability of the sources and potential difficulties to eliminate the batch-to-batch variations of wild sources [10] are still limiting the spread of those materials in the health industry [10]. Moreover, the lower denaturation temperatures may also be hindering market products due to processing bottlenecks. Nevertheless some marine-derived collagens have been used to produce materials for tissue engineering and regenerative medicine and drug delivery. In Table 35.2, we will provide a list with some examples of products based on marine collagens that have been validated at the lab level.

Despite the fact that gelatine is still a niche product, it is gaining popularity in the food industry given both its properties and the apparent compliance with Muslim and Jewish religious requirements, contrary to the pig and cow derived gelatines. Marine derived gelatine can be roughly divided into two different groups: gelatine obtained from cold water fish species and gelatine obtained from warm water species. Indeed, the origin of the material is mainly responsible for the gelation properties and dictates the possible uses of gelatine (see [12] for extensive

Table 35.2 Example of products based on marine collagens developed in research lab context. BR: bone regeneration; HA = hydroxyapatite; SR = skin regeneration; OMR = oral mucosa regeneration; CR = cartilage repair; WH = wound healing; GTR = guided tissue regeneration; DD = drug delivery.

Target	Product	Composition	Source	Ref
BR	Scaffold	Coll Type I/HA	*Fish*	[127]
BR	Scaffold	Coll Type I/Chitosan	*Fish*	[128]
BR	Scaffold	Coll Type I/Chitosan/HA	*Shark*	[129]
BR	Scaffold	Type I	*Shark*	[42]
SR	Scaffold	Coll Type I/Elastin	*Fish*	[130]
OMR	Scaffold	Coll Type I/Chitosan	*Fish*	[131]
CR	Scaffold	Coll Type II	*Jellyfish*	[92]
WH	Hydrogel	Coll Type I	*Cuttlefish*	[132]
SR	Scaffold	Coll Type I/Alginate/ Chitoligosaccarides	*Fish*	[133]
GTR	Membrane	Type I	*Echinoderm*	[94]
DD	Particles	Type I	*Sponge*	[134]
DD	Coating	Type I	*Sponge*	[135]
DD	Particles	Type I	*Sponge*	[136]

review). Gelatine obtained from warm water species has a slightly lower gelling temperature in comparison to those obtained from mammalian sources, thus possibly simplifying the industrial production of some foods [137] while also providing a better aroma release [138]. Gelatine produced using cold-water species has a lower gelling point and is currently used in the pharmaceutical/nutraceutical industry to encapsulate several compounds [137].

Fish collagenous peptides have been also receiving attention, mainly being used as a component of dietary integrators and skin creams. Collagenous peptides extracted from different fish species have shown a wide range of activities on different physiological and pathological processes (see [137] for a detailed review) thus representing a valuable compound for functional foods. They have antioxidant activity as well as antihypertensive activity, thus representing a safe and promising alternative to other pharmaceutical compounds, which in turn might result in allergic reactions or undesired effects [137]. The possible benefits of collagenous peptides as components in functional food has been demonstrated also in their capabilities to reduce joint pain deriving from both osteoarthritis and osteoporosis by acting on the causes of cartilage degeneration and on the regeneration processes [137]. Collagenous peptides have also demonstrated a promising antimicrobial activity, the capability to modulate calciotropic activity, to potentially interact with the satiety feedback hormonal loop and protect the gastric mucosa, regulate glucose and lipid metabolisms, inhibit the coagulation cascade and promote the wound healing process [137]. More recently, collagenous peptides obtained from *Tilapia* sp. have shown the striking capability to induce *in vitro* the differentiation of mesenchymal stem cells into bone lineages [139], thus increasing the potential use of these small amino acidic sequences obtained from aquatic organisms.

35.7 3D Printing as a Future Trend in the Application of Marine Collagens

35.7.1 3D-Printing Technology is a Promising Tool with Many Burdens

A relative new technology has appeared in the last decade and might have a significant impact on the huge need of biosubstitutes mimicking human tissues for research, diagnostic and therapeutic purposes: 3D printing [140,141], a processing technique for controlled computer-assisted design and build-up that promises patient-specific 3D bioconstructs.

Bioprinting, a particular 3D printing approach that includes the presence of live cells during the microfabrication steps, is much more challenging. Indeed it requires a whole strategy compatible with the presence of cells during printing, during materials phase transition as well as an adequate micro/nano-environment to further sustain cells viability and growth.

Many bioprinters are currently available in the market and can be consulted elsewhere [142,143] (e.g. from MicroFab, Organovo, EnvisionTec, Regenovo and RegenHU); however no *one-size-fits-all* bioprinter exists [144,145] since bioinks – the specific materials use to build the extra-cellular scaffolding – requirements remarkably vary with the type of printer [146]. Micro-extrusion-based bioprinters deposit filaments/drops of high cell density demanding highly viscous, shear thinning and thixotropic polymers as bioinks. In general, such high viscous polymers require higher pressures to be applied for biomaterial dispensing, but the resultant high stress can jeopardize and significantly reduce cell viability [147]. Nevertheless, extrusion printers are generally more versatile given the possibility to use high cell densities and build larger and clinical size relevant constructs in less time, thus reducing the exposure time to unfavorable cell environment. Inkjet and laser-based machines print single droplets of low viscosity bioinks at higher resolution

than extrusion printers. However, the former are limited in terms of cell density and to chemical and photocurable materials, while extrusion based devices can support materials phase transition mediated by chemical, photo-crosslinking, sheer thinning and temperature [146].

The list of available biomaterials for bioprinting is still limited (alginate, hyaluronic acid and a few more [142]) and their rheological properties must be often adjusted in order to meet the specific requirements of the printing process. This is generally achieved by mixing the selected biopolymer with viscosity enhancers or other shear thinning polymers [148]. Besides, many other aspects still require solutions in order to overcome relevant drawbacks: (i) a strategy to obtain bigger constructs [149]; (ii) a reliable method to include channels and/or promote a functional vasculature [150,151]; (iii) develop a mechanically tunable and bioactive printable bioinks [148,152].

35.7.2 3D-Printing of Collagen

Collagen-based bioinks, due to the collagen specific features, have the potentiality of generate a cell instructing micro/nano-environment with a wide-range of possible tissue engineering applications. As mentioned in the previous sections, the phase transition of collagen can be triggered by gelation with temperature and pH, by chemical crosslinking or following the complexation with oppositely charged polymers/proteins. Denaturated collagen is also being modified with methacrylate moieties to control the phase transition by photo-initiation with UV or visible light (e.g. rhCollagen provided by CollPlant). Besides, gelatine is also being explored using the same strategy, with methacrylated gelatine, known commonly as GelMA, being explored by many research groups across the globe.

As many other polymers, the viscosity of collagen bioinks can be adjusted by: (i) changing the concentration; (ii) using a different source of collagen; (iii) changing the temperature and the pH; (iv) varying cell density. According to the range of viscosities and phase transition methods, theoretically, collagen-based bioinks could be used with any type of printer. However, from now on we will mainly focus on the extrusion-based printing method for collagen or its derived forms.

Conceiving a printable and cell compatible bioink requires understanding the bioprinter concept and to carefully consider at least the following listed parameters.

35.7.2.1 Rheology: Viscosity and Thixotropic Behavior

The bioink rheological properties dictate the successful bioprinting strategy being accountable for the injectability, shear stress generated upon printing, fidelity and resolution of the construct, cell viability and cell fate. Ideal bioinks, either composed of a single component or a blend of different materials, should have a marked shear thinning behavior in order to easily flow out the nozzle and prevent a loss of cell viability [153]. It has been reported that the rheological properties of gelatine/sodium alginate blends tend to change with time [154], eventually justified by the formation of a more organized and thixotropic polymer blend.

35.7.2.2 Printing and Phase Transition Settings

Increasing construct resolution by using a nozzle with small diameter comes with the disadvantage of a simultaneous increase of the shear stress applied to the cells, limiting cell viability, during the printing process [147]. To mitigate this, the applied pressure to force the material/cells passing from the nozzle has to be decreased and consequently the printing time increases. However, the printing duration and cartridge temperature are parameters that have also been pointed out as affecting cell viability [154]. A compromise between parameters, defined for each case, needs to be established.

35.7.2.3 Post-Printing and Upon Culture/Implantation Stability

With the purpose of building 3D bioconstructs with high fidelity to the designed dimensions and geometries, which is crucial for future patient-specific implantable devices, the bioink must be stable within its functional timeframe. This requires optimization of properties to avoid loss of shape of the struts and control over gel properties such as swelling and shrinkage, as well as over degradation rate [155].

Given all these limitations, 3D printing technology using collagen has been applied more effectively to produce acellular scaffolds. For instance, Lee et al. [156] reported the deposition of layers of vascular endothelial growth factor-rich fibrin gel and rat tail collagen type I (BD Biosciences), nebulizing the printing area with sodium bicarbonate solution to trigger collagen jellification. Some other studies have reported the use of highly viscous solutions and stabilizing the structure by chemical-crosslinking. Shin et al. [157] have prepared atelocollagen type I acellular scaffolds using a three-step processing approach: 3D printing coupled with precipitation at high pH followed by dehydration and chemical crosslinking to stabilize the structure [157] – see Figure 35.2a,b. Also using a multi-step approach, Lode et al. [158] printed, freeze-dried and gamma-irradiated collagen extracted from porcine skin (Figure 35.2c), producing robust scaffolds with at least 33 layers. In their turn, Inzana et al. [159] reported the low temperature 3D printing of calcium phosphate formulations supplemented with 1–2% bovine skin collagen type I.

To the best of our knowledge, 3D printing of marine origin collagens has not yet been successfully achieved.

35.7.3 Collagen Bioprinting Progresses

Despite the bottlenecks associated with bioprinting that were briefly discussed in the previous paragraphs, some attempts are being carried out using collagens, walking the (long) way up to a cell-laden printed construct.

Different researchers have explored the versatility of multimaterials printing, combining hydrogels with polycaprolactone (PCL) to obtain more robust 3D structures with laden growth factors aiming at different tissue engineering applications [160–162]. One of the studies has reported the use of neutralized atelocollagen (Koken) to encapsulate endothelial and liver cells struts in between PCL [160], promoting collagen gelation by incubation at 37°C in a 5% CO_2 atmosphere, a cell-friendly approach. Seeking an osteochondral application [162], three different bioinks were co-printed in-between PCL struts: atelocollagen + bone-morphogenetic protein 2 (BMP2) + human turbinate-derived mesenchymal stromal cells (hTMS); transforming growth factor-beta (TGF-beta) + MonoCB[6]-hyaluronic acid; and acellular 1,6-diaminohexane-hyaluronic acid [161]. The authors have successfully printed the hydrogels/PCL in different layers, which resulted in outstanding reconstruction of osteochondral tissue in the knee joints of rabbits. The same approach was explored for bone tissue engineering. In the third study, human dental pulp stem cells were co-bioprinted in two different bioinks in between PCL struts: alginate/gelatine blend loaded with vascular endothelial growth factor (VEGF) and atelocollagen loaded with BMP2 [161]. Moreover, Wu et al. [163] have developed bioinks containing collagen and gelatine, together with human corneal epithelial cells (HCECs) and supplemented with sodium citrate and alginate. By adjusting the citrate/alginate ratio, it was possible to tune up hydrogel degradation over time, also achieving cell viability over 90%.

Yeo et al. [164] reported an innovative 3D bioprinting method to develop core-shell alginate-collagen hydrogel using a core-sheath nozzle. The core region was composed by collagen type I from tendon porcine, whereas the shell was made of alginate crosslinked during the printing by a calcium chloride aerosol. Their system allowed obtaining higher mechanical stability, retaining collagen inside, a +10% shift in viability of human adipose

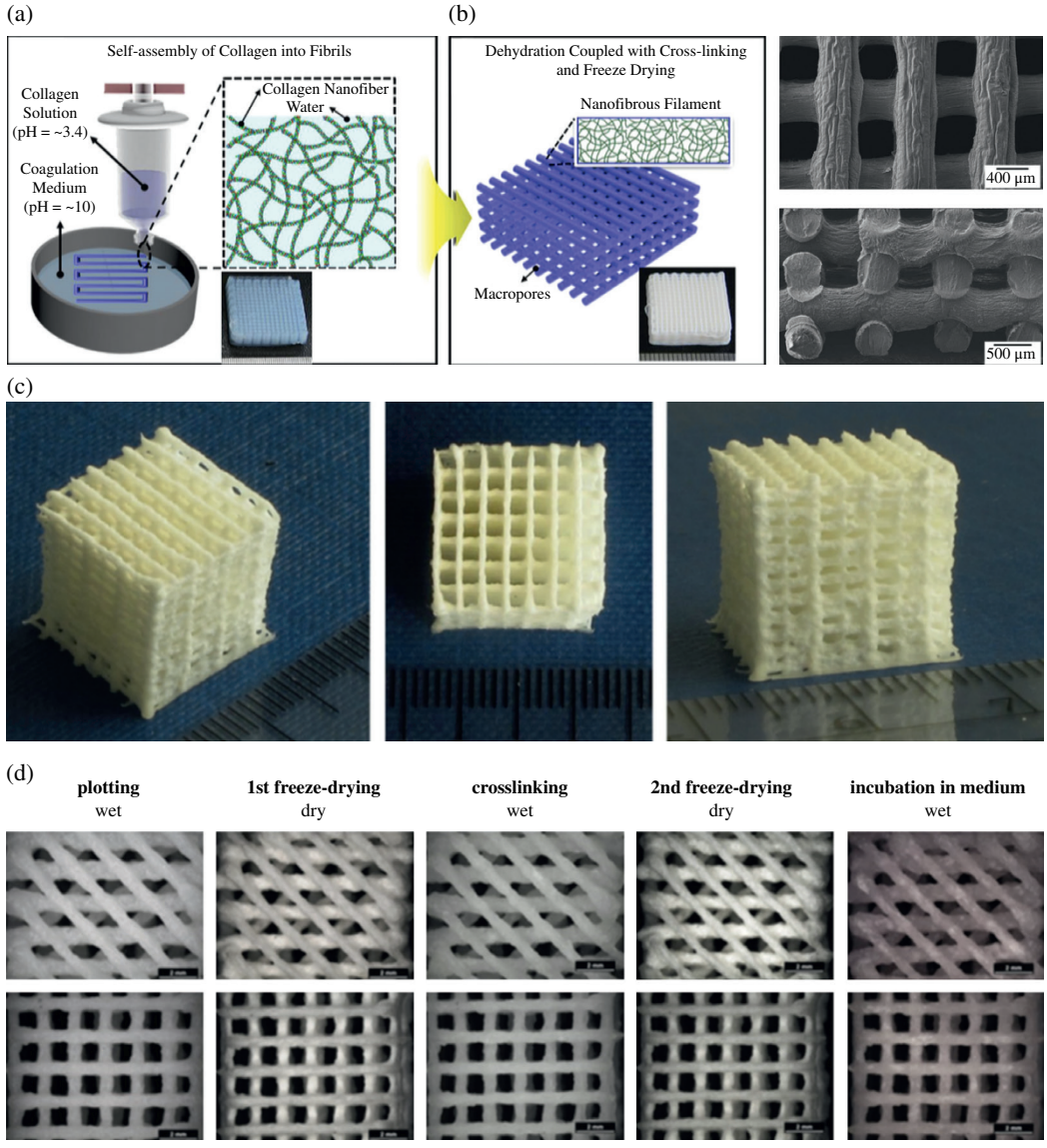

Figure 35.2 Examples of step-wise preparation of collagen printed 3D scaffolds. (a, b) Printing collagen in pH 10 coagulation bath followed by chemical crosslinking and freeze-drying, adapted from [157]. (c) 3D printed scaffolds of collagen type I with 33 layers (1.5 mm width cube, with orthogonal adjacent layers); (d) stereo light microscopy images of 3D printed collagen scaffolds in consecutive production steps (filaments of adjacent layers forming an angle of 45° (upper panel) or 90° (lower panel); reproduced with permission from [158].

derived stem cells (hASCs) and higher hepatogenic differentiation efficiency with respect to 100% alginate hydrogel. The same research group has investigated blends of collagen/ECM and alginate using a new approach for the bioprinting procedure using MC3T3-E1 pre-osteoblasts cells and hASCs for hepatogenic differentiation [165]. In contrast with standard procedures, cells were firstly cultured onto porcine tendon collagen type I and only after was alginate added. With this pre-culture step, the printability of collagen was improved, while the addition of alginate enabled the control of biodegradation.

Hinton et al. [166] have disclosed a new approach for printing soft proteins and polysaccharide hydrogels, with increased mechanical properties while providing complex 3D internal structures and tailored external anatomical designs. The trick consisted of printing the hydrogel in a bath of a thermo-reversible support made of suspended gelatine microparticles that exhibited a Bingham plastic-like behavior. The particles behaved as a rigid body at low shear stress and as viscous fluid at high shear stress, thus facilitating the dispensing of the bioink in between the particles while keeping high printing fidelity level thanks to the high thixotropic response of the gelatin particles. The efficacy of this procedure is illustrated in Figure 35.3, where 9 identical bifurcated tubes printed with fibrin (center) and 7 of those bifurcated tubes and 1 femur obtained by spontaneous gelation of rat tail collagen type I (right) can be seen.

Highly concentrated collagen type I extracted form rat-tail can be also printed without the use of supporting baths, as was shown by Rhee and coworkers [167]. A viscous solution of solubilized collagen was neutralized with concentrated sodium hydroxide and PBS, a high concentration of primary fibrochondrocytes was added and the printability was assessed by producing a 3D sheep meniscus (Figure 35.4).

The biochemical potential of collagen regarding its biomedical use would probably be maximized if the secondary and tertiary structures could be preserved, and the crosslinked collagen fibrils suspended in neutral buffer with cell compatible crosslinker; this may be a promising field. So far only one method has been fully validated, based on the riboflavin/UVB crosslinking [168]. It has been demonstrated that several proteins are able to induce the fibril assembling/disassembling in echinoderms [169–173] and marine sponges [80]. Although the exact mechanisms of such natural reversible crosslinking are still not fully understood, they could serve as bioinpiration for the development of new strategies for the *in vitro* production of cell-laden 3D collagenous hydrogels, including injectable systems. In this view, it might be worth exploring the potentialities of these phenomena in 3D-printing, where the need of a suitable material and crosslinking strategy has yet to be developed [144]. Marine origin materials (collagens and associated compounds) may hence have not a supporting, but instead the best role, in collagen-based bioprinted constructs for regenerative medicine.

35.8 Concluding Remarks

Collagen is a versatile building block biopolymer and marine collagens have demonstrated to be valuable and alternative materials. So far the

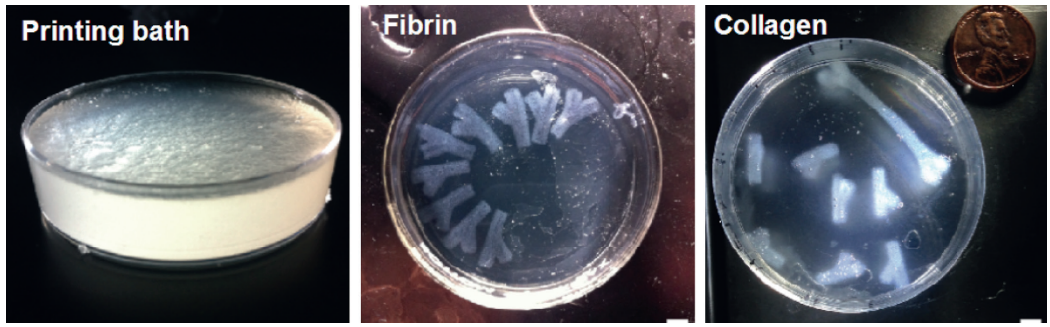

Figure 35.3 (Left) Printing bath composed of microparticles of gelatine with Bingham plastic behavior. (Center) 9 fibrin-based identical bifurcated tubes were produced by 3D printing and trombin crosslinking. (Right) 7 identical bifurcated tubes and 1 small femur model obtained with collagen-based ink. Scale bar: 5 mm. Images adapted with permission from Hinton et al. (2015) [166].

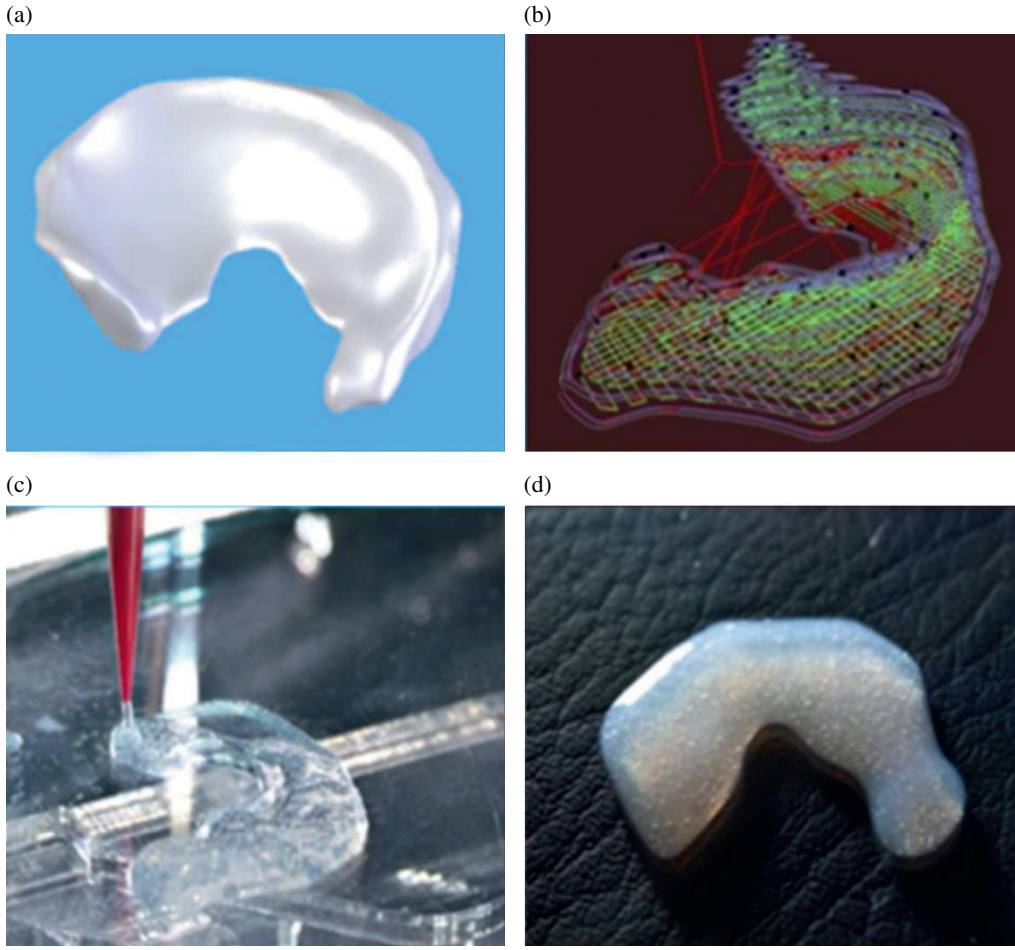

Figure 35.4 Printing of sheep meniscus with rat-tail collagen type I. (a) view of reconstructed computerized tomography scans; (b) printing path; (c) 3D hydrogel during the printing step; (d) 3D printed meniscus after collagen gelation. Images reproduced from Rhee et al. [167] with permission from ACS Publications.

huge potential and diversity of marine species that might be used as a source of native collagen, gelatine or collagenous peptides is still far from being explored. While in the coming years further descriptions of extraction processes and characterizations of marine fish collagens will probably slow down, a significant increment of publications, aimed to show the potential applications of collagen and collagen derived molecules, is expected. Most of the research on marine collagen has been so far focused on fish, given their abundance and the need to extract value from the byproducts generated during their industrial processing. Despite the fact that invertebrates have shown the presence of interesting and unique collagens, they have received far less attention and there have been very few studies aimed to evaluate their potentialities as a source of innovative collagens. This is possibly the result of concerns regarding the availability/sustainability of the sources or related to the costs associated with the collection/farming that have discouraged further investigations aimed to demonstrate their suitability as a material for producing biomedical products. Whether collagens for wound healing or tissue regeneration in general, gelatines may be used in the nutraceutical and pharmaceutical industries or

collagenous peptides as bioactive compounds with therapeutic interest. Nevertheless, some species, such as some echinoderms or cnidarians, can be easily farmed and the characteristics of their collagens might bring substantial advantages justifying higher extraction/purification costs. In this view, the few preliminary investigations present in literature showed promising results.

Currently, scientists are conducting experiments aiming to fully demonstrate, in a clinical environment, that marine collagens can compete with mammalian collagen both in terms of performance and safety. The next step will require the establishment of industrial strategies to provide suitable collagen with a competitive prize. It is thus crucial that one of the available concepts (or a new one arising) evolves from business-to-business to business-to-consumer, i.e., reaching the clinics as a milestone of the biomedical relevance of marine collagens. Regarding the case of collagens derived from rare or difficult-to-access species, such as marine sponges, it is expected that new mariculture or land-based farming strategies will be developed following the pioneer work of companies as Porifarma in the Netherlands and Turkey and Studio Associato GAIA in Italy.

Wrapping up, one foresees that during the next few decades fish gelatine will increase its market share and gradually substitute mammalian gelatine while the research on collagenous peptides as well as fish collagens will undergo scrutiny to fully demonstrate their potential as marketable biomedical products. In parallel, at a research level, more unique collagens obtained from invertebrates might reach the bench validation and/or provide clues for alternative crosslinking strategies.

References

1 Shoulders, M.D., Raines, R.T. (2009) Collagen structure and stability. *Annual Review of Biochemistry.* 78 (1), 929–958.

2 Exposito, J.Y., Valcourt, U., Cluzel, C. et al. (2010) The fibrillar collagen family. *International Journal of Molecular Science.* 11, 407–426.

3 Wilkie, I.C., Fassini, D., Cullorà, E. et al. (2015) Mechanical properties of the compass depressors of the sea-urchin *Paracentrotus lividus* (Echinodermata, Echinoidea) and the effects of enzymes, neurotransmitters and synthetic tensilin-like protein. *PLoS One.* 10 (3), e0120339.

4 Di Lullo, G., Sweeney, S., Korkko, J. et al. (2001) Mapping the ligand-binding sites and disease-associated mutations on the most abundant protein in the human, type i collagen. *Journal of Biological Chemistry.* 277 (6), 4223–4231.

5 Pankov, R., Yamada, K.M. (2002) Fibronectin at a glance. *Journal of Cell Science.* 115 (20), 3861–3863.

6 Gelse, K. (2003) Collagens-structure, function, and biosynthesis. *Advanced Drug Delivery Reviews.* 55 (12), 1531–1546.

7 Schuppan, D., Schmid, M., Somasundaram, R. et al. (1998) Collagens in the liver extracellular matrix bind hepatocyte growth factor. *Gastroenterology.* 114 (1), 139–152.

8 Czirok, A., Rongish, B., Little, C. (2004) Extracellular matrix dynamics during vertebrate axis formation. *Developmental Biology.* 268 (1), 111–122.

9 Bailey, A., Paul, R., Knott, L. (1998) Mechanisms of maturation and ageing of collagen. *Mechanisms of Ageing and Development.* 106 (1), 1–56.

10 Silva, T. H., Moreira-Silva, J., Marques, A. et al. (2014) Marine origin collagens and its potential applications. *Marine Drugs.* 12 (12), 5881–5901.

11 Keane, J., Badylak, F. (2014) Biomaterials for tissue engineering applications. *Seminar in Pediatric Surgery.* 23, 112–118.

12 Karim, A., Bhat, R. (2009) Fish gelatin: properties, challenges, and prospects as an alternative to mammalian gelatins. *Food Hydrocolloids.* 23 (3), 563–576.

13 Haug, I.J., Draget, K.I., Smidsrød, O. (2014) Physical and rheological properties of fish gelatin compared to mammalian gelatin. *Food Hydrocolloids.* 18 (2), 203–213.

14 Gómez-Guillén, M.C., Giménez, B., López-Caballero, M.E. et al. (2011) Functional and bioactive properties of collagen and gelatin from alternative sources: A review. *Food Hydrocolloids.* 25 (8), 1813–1827.

15 Mora, C., Tittensor, D., Adl, S. et al. (2011) How many species are there on earth and in the ocean?. *PLoS Biology.* 9 (8), e1001127.

16 Appeltans, W., Ahyong, S., Anderson, G. et al. (2012) The magnitude of global marine species diversity. *Current Biology.* 22 (23), 2189–2202.

17 Wasswa, J., Tang, J., Gu, X. (2007) Utilization of fish processing by-products in the gelatin industry. *Food Reviews International.* 23 (2), 159–174.

18 Nagai, T., Worawattanamateekul, W., Suzuki, N. et al. (2000) Isolation and characterization of collagen from rhizostomous jellyfish (*Rhopilema asamushi*). *Food Chemistry.* 70 (2), 205–208.

19 Sivakumar, P., Suguna, L., Chandrakasan, G. (2000) Molecular species of collagen in the intramuscular connective tissues of the marine crab, *Scylla serrata*. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology.* 125 (4), 555–562.

20 Addad, S., Exposito, J., Faye, C. et al. (2011) Isolation, characterization and biological evaluation of jellyfish collagen for use in biomedical applications. *Marine Drugs.* 9 (12), 967–983.

21 Fleury, C., Serpentini, A., Kypriotou, M. et al. (2011) Characterization of a non-fibrillar-related collagen in the mollusc *Haliotis tuberculata* and its biological activity on human dermal fibroblasts. *Marine Biotechnology.* 13 (5), 1003–1016.

22 Di Benedetto, C., Barbaglio, A., Martinello, T. et al. (2014) Production, characterization and biocompatibility of marine collagen matrices from an alternative and sustainable source: the sea urchin *Paracentrotus lividus*. *Marine Drugs.* 12 (9), 4912–4933.

23 Ahmad, M., Benjakul, S. (2010) Extraction and characterisation of pepsin-solubilised collagen from the skin of unicorn leatherjacket (*Aluterus monocerous*). *Food Chemistry.* 120 (3), 817–824.

24 Ogawa, M., Portier, R., Moody, M. et al. (2004) Biochemical properties of bone and scale collagens isolated from the subtropical fish black drum (*Pogonia cromis*) and sheepshead seabream (*Archosargus probatocephalus*). *Food Chemistry.* 88 (4), 495–501.

25 Veeruraj, A., Arumugam, M., Balasubramanian, T. (2013) Isolation and characterization of thermostable collagen from the marine eel-fish (*Evenchelys macrura*). *Process Biochemistry.* 48 (10), 1592–1602.

26 Żelechowska, E., Sadowska, M., Turk, M. (2010) Isolation and some properties of collagen from the backbone of Baltic cod (*Gadus morhua*). *Food Hydrocolloids.* 24 (4), 325–329.

27 Yu, D., Chi, C., Wang, B. et al. (2014) Characterization of acid-and pepsin-soluble collagens from spines and skulls of skipjack tuna (*Katsuwonus pelamis*). *Chinese Journal of Natural Medicines.* 12 (9), 712–720.

28 Senaratne, L., Park, P., Kim, S. (2006) Isolation and characterization of collagen from brown backed toadfish (*Lagocephalus gloveri*) skin. *Bioresource Technology.* 97 (2), 191–197.

29 Jongjareonrak, A., Benjakul, S., Visessanguan, W. et al. (2005) Isolation and characterisation of acid and pepsin-solubilised collagens from the skin of Brownstripe red snapper (*Lutjanus vitta*). *Food Chemistry.* 93 (3), 475–484.

30 Ciarlo, A., Paredi, M., Fraga, A. (1997) Isolation of soluble collagen from hake skin (*Merluccius hubbsi*). *Journal of Aquatic Food Product Technology.* 6 (1), 65–77.

31 Matmaroh, K., Benjakul, S., Prodpran, T. et al. (2011) Characteristics of acid soluble collagen and pepsin soluble collagen from scale of spotted golden goatfish (*Parupeneus heptacanthus*). *Food Chemistry.* 129 (3), 1179–1186.

32 Nalinanon, S., Benjakul, S., Kishimura, H. (2010) Collagens from the skin of arabesque greenling (*Pleurogrammus azonus*) solubilized with the aid of acetic acid and pepsin from albacore tuna (*Thunnus alalunga*) stomach. *Journal of the Science of Food and Agriculture.* 90 (9), 1492–1500.

33 Ogawa, M., Portier, R., Moody, M. et al. (2004) Biochemical properties of bone and scale collagens isolated from the subtropical fish black drum (*Pogonia cromis*) and sheepshead seabream (*Archosargus probatocephalus*). *Food Chemistry.* 88 (4), 495–501.

34 Noitup, P., Ganjanagoonchorn, W., Morrissey, M. (2005) Characteristic comparison of albacore tuna (*Thunnus alalunga*) and silver-line grunt (*Pomadasys kaakan*). *Journal of Aquatic Food Product Technology.* 14 (1), 17–28.

35 Nalinanon, S., Benjakul, S., Visessanguan, W. et al. (2007) Use of pepsin for collagen extraction from the skin of bigeye snapper (*Priacanthus tayenus*). *Food Chemistry.* 104 (2), 593–601.

36 Yunoki, S., Nagai, N., Suzuki, T. et al. (2004) Novel biomaterial from reinforced salmon collagen gel prepared by fibril formation and cross-linking. *Journal of Bioscience and Bioengineering.* 98 (1), 40–47.

37 Wang, L., An, X., Xin, Z. et al. (2002) Isolation and characterization of collagen from the skin of deep-sea redfish (*Sebastes mentella*). *Journal of Food Science.* 72 (8), 450–455.

38 Nagai, T., Araki, Y., Suzuki, N. (2002) Collagen of the skin of ocellate puffer fish (*Takifugu rubripes*). *Food Chemistry.* 78 (2), 173–177.

39 Yan, M., Li, B., Zhao, X. et al. (2008) Characterization of acid-soluble collagen from the skin of walleye pollock (*Theragra chalcogramma*). *Food Chemistry.* 107 (4), 1581–1586.

40 Kaewdang, O., Benjakul, S., Kaewmanee, T. et al. (2014) Characteristics of collagens from the swim bladders of yellowfin tuna (*Thunnus albacares*). *Food Chemistry.* 155, 264–270.

41 Kittiphattanabawon, P., Benjakul, S., Visessanguan, W. et al. (2010) Isolation and characterization of collagen from the cartilages of brownbanded bamboo shark (*Chiloscyllium punctatum*) and blacktip shark (*Carcharhinus limbatus*). *Food Science and Technology.* 43 (5), 792–800.

42 Fernandes-Silva, S., Moreira-Silva, J., Silva, T. et al. (2013) Porous hydrogels from shark skin collagen crosslinked under dense carbon dioxide atmosphere. *Macromolecular Bioscience.* 13 (11), 1621–1631.

43 Tan. C., Karim, A., Latiff, A. et al. (2013) Extraction and characterization of pepsin-solubilized collagen from the body wall of crown-of-thorns starfish (*Acanthaster planci*). *International Food Research Journal.* 20 (6), 3013–3020.

44 Nagai, T., Suzuki, N. (2000) Partial characterization of collagen from purple sea urchin (*Anthocidaris crassispina*) test. *International Journal of Food Science and Technology.* 35 (5), 497–501.

45 Kimura. S., Omura, Y., Ishida, M., et al. (1993) Molecular characterization of fibrillar collagen from the body wall of starfish *Asterias amurensis. Comparative Biochemistry and Physiology Part B: Comparative Biochemistry.* 104 (4), 663–668.

46 Adibzadeh, N., Aminzadeh, S., Jamili, S. et al. (2014) Purification and characterization of pepsin-solubilized collagen from skin of sea cucumber *Holothuria parva*. *Applied Biochemistry and Biotechnology.* 173 (1), 143–154.

47 Liu, Z., Oliveira, A., Su, Y. (2010) Purification and characterization of pepsin-solubilized collagen from skin and connective tissue of giant red sea cucumber (*Parastichopus*

californicus). *Journal of Agricultural and Food Chemistry.* 58 (2), 1270–1274.

48 Zhong, M., Chen, T., Hu, C. et al. (2015) Isolation and characterization of collagen from the body wall of sea cucumber *Stichopus monotuberculatus*. *Journal of Food Science.* 80 (4), 671–679.

49 Cui, F., Xue, C., Li, Z. et al. (2007) Characterization and subunit composition of collagen from the body wall of sea cucumber *Stichopus japonicus*. *Food Chemistry.* 100 (3), 1120–1125.

50 Abedin, M., Karim, A., Ahmed, F. et al. (2012) Isolation and characterization of pepsin-solubilized collagen from the integument of sea cucumber (*Stichopus vastus*). *Journal of the Science of Food and Agriculture.* 93 (5), 1083–1088.

51 Nagai, T., Suzuki, N. (2002) Preparation and partial characterization of collagen from paper nautilus (*Argonauta argo*, Linnaeus). *Food Chemistry.* 76 (2), 149–153.

52 Nagai, T., Nagamori, K., Yamashita, E. et al. (2002) Collagen of octopus *Callistoctopus arakawai* arm. *International Journal of Food Science & Technology.* 37 (3), 285–289.

53 Coelho, R.C.G., Marques, A., Oliveira, S. et al. (2017) Extraction and characterization of collagen from Antarctic and Sub-Antarctic squids and its potential application in tissue engineering hybrid scaffolds. *Materials Science and Engineering C: Materials for Biological Applications.* 78, 787–795.

54 Nagai, T., Yamashita, E., Taniguchi, K. et al. (2001) Isolation and characterisation of collagen from the outer skin waste material of cuttlefish (*Sepia lycidas*). *Food Chemistry.* 72 (4), 425–429.

55 Krishnamoorthi, J., Ramasamy, P., Shanmugam, V. et al. (2017) Isolation and partial characterization of collagen from outer skin of *Sepia pharaonis* (Ehrenberg, 1831) from Puducherry coast. *Biochemistry and Biophysic Reports.* 10, 39–45.

56 Bairati, A., Gioria, M. (2004) Collagen fibrils of an invertebrate (*Sepia officinalis*) are heterotypic: immunocytochemical demonstration. *Journal of Structural Biology* 147 (2), 159–165.

57 Nam, K., You, S., Kim, S. (2008) Molecular and physical characteristics of squid (*Todarodes pacificus*) skin collagens and biological properties of their enzymatic hydrolysates. *Journal of Food Science.* 73 (4), 249–255.

58 Zhang, J., Duan, R., Huang, L. et al. (2014) Characterisation of acid-soluble and pepsin-solubilised collagen from jellyfish (*Cyanea nozakii* Kishinouye). *Food Chemistry.* 150, 22–26.

59 Heinemann, S., Ehrlich, H., Douglas, T. et al. (2007) Ultrastructural studies on the collagen of the marine sponge *Chondrosia reniformis* Nardo. *Biomacromolecules.* 8 (11), 3452–3457.

60 Pallela, R., Bojja, S., Janapala, V. (2011) Biochemical and biophysical characterization of collagens of marine sponge, *Ircinia fusca* (Porifera: Demospongiae: Irciniidae). *International Journal of Biological Macromolecules.* 49 (1), 85–92.

61 Tziveleka, L.-A., Ioannou, E., Tsiourvas, D. et al. (2017) Collagen from the marine sponges *Axinella cannabina* and *Suberites carnosus*: isolation and morphological, biochemical, and biophysical characterization. *Marine Drugs.* 15(6), 152.

62 Jeevithan, E, Bao, B., Bu, Y. et al. (2014) Type II collagen and gelatin from silvertip shark (*Carcharhinus albimarginatus*) cartilage: isolation, purification, physicochemical and antioxidant properties. *Marine Drugs.* 12 (7), 3852–3873.

63 Moreira-Silva, J.C. et al., 2015 Patent WO 2015186118 A1.

64 Hwang, J.H., Mizuta, S., Yokoyama, Y. et al. (2007) Purification and characterization of molecular species of collagen in the skin of skate (*Raja kenojei*). *Food Chemistry* 2007, 100, 921.

65 Shimizu, K., Amemiya, S., Yoshizato, K. (1990) Biochemical and immunological characterization of collagen molecules from

echinothurioid sea urchin *Asthenosoma ijimai*. *Biochimica et Biophysica Acta (BBA) – Protein Structure and Molecular Enzymology.* 1038 (1), 39–46.

66 Uriarte-Montoya, M., Arias-Moscoso, J., Plascencia-Jatomea, M. et al. (2010) Jumbo squid (*Dosidicus gigas*) mantle collagen: Extraction, characterization, and potential application in the preparation of chitosan/collagen biofilms. *Bioresource Technology.* 101 (11), 4212–4219.

67 Palpandi, C., Ramasamy, P., Rajinikanth, T. et al. (2010) Extraction of collagen from mangrove archeaogastropod *Nerita* (*Dostia*) *crepidularia* Lamarck, 1822. *American-Eurasian Journal of Scientific Research*. 5(1), 23–30.

68 Sivakumar, P., Suguna, L, Chandrakasan, G. (1997) Purification and partial characterization of a type V like collagen from the muscle of marine prawn, *Penaeus indicus*. *Journal of Bioscience.* 22 (2), 131–141.

69 Krishnan, S., Perumal, P. (2006) Preparation and biomedical charcterization of jellyfish (*Chrysaora quinquecirrha*) collagen from southeast cost of India. *International Journal of Pharmacy and Pharmaceutical Science*. 5 (3), 698–701.

70 Song, E., Yeon Kim, S., Chun, T. et al. (2006) Collagen scaffolds derived from a marine source and their biocompatibility. *Biomaterials.* 27 (15), 2951–2961.

71 Moreira-Silva, J., Diogo, S.G., Marques, A.L.P. et al. (2017) Marine Collagen isolation and processing envisaging biomedical applications. In: *Biomaterials from Nature for Advanced Devices and Therapies* (eds N.M. Neves, Rui L. Reis). John Wiley & Sons, Inc., Hoboken, New Jersey.

72 Sadowska, M., Kołodziejska, I., Niecikowska, C. (2003) Isolation of collagen from the skins of Baltic cod (*Gadus morhua*). *Food Chemistry.* 81, 257–262.

73 Sadowska, M., Kołodziejska, I. (2005) Optimisation of conditions for precipitation of collagen from solution using κ-carrageenan. Studies on collagen from the skin of Baltic cod (*Gadus morhua*). *Food Chemistry.* 91, 45–49.

74 Barros, A., Aroso, I., Silva, T. et al. (2015) Water and carbon dioxide: green solvents for the extraction of collagen/gelatin from marine sponges. *ACS Sustainable Chemistry & Engineering.* 3 (2), 254–260.

75 Silva, J., Barros, A., Aroso, I. et al. (2016) Extraction of collagen/gelatin from the marine demosponge *Chondrosia reniformis* (Nardo, 1847) using water acidified with carbon dioxide – process optimization. *Industrial & Engineering Chemistry Research.* 55, 6922–5930.

76 Birk, D.E., Nurminskaya, N.V., Zycband, E.L. (1995) Collagen fibrillogenesis in situ: fibril segments undergo post-depositional modifications resulting in linear and lateral growth during matrix development. *Developmental Dynamics.* 202, 229–243.

77 Trotter, J.A., Lyons-Levy, G., Luna, D. et al. (1996) Stiparin: a glycoprotein from sea cucumber dermis that aggregates collagen fibrils. *Matrix Biology.* 15, 99–110.

78 Muyonga, J. Cole, C., Duodu, K. (2004) Characterisation of acid soluble collagen from skins of young and adult Nile perch (*Lates niloticus*). *Food Chemistry.* 85, 81–89.

79 Wilkie, I.C. (2005) Mutable collagenous tissue: overview and biotechnological perspective. *Progress in Molecular and Subcellular Biology.* 39, 221–250.

80 Fassini, D., Parma, L., Lembo, F. et al. (2014) The reaction of the sponge *Chondrosia reniformis* to mechanical stimulation is mediated by the outer epithelium and the release of stiffening factor(s). *Zoology.* 117 (4), 282–291.

81 Swatschek, D., Schatton, W., Kellermann, J. et al. (2002) Marine sponge collagen: isolation, preparation and effects on the skin parameters surface-pH, moisture and sebum, *European Journal of Pharmaceutics and Biopharmaceutics.* 53, 107–113.

82 Kolodziejska, I., Sikorski E.Z., Niecikowska C. (1999) Parameters affecting the isolation

of collagen from squid (*Illex argentinus*) skins. *Food Chemistry.* 66 (2), 153–157.

83 Ngo, D., Vo, T., Ngo, D. et al. (2012) Biological activities and potential health benefits of bioactive peptides derived from marine organisms. *International Journal of Biological Macromolecules.* 51 (4), 378–383.

84 Booz, M. (2001) Electrophoresis. In: *Molecular Biology Problem Solver: A Laboratory Guide* (ed. A.S. Gerstein). John Wiley & Sons, Inc., New York, USA, pp. 331–371.

85 Møller, H., Poulsen, J. (2009) Staining of glycoproteins/proteoglycans on SDS-Gels. In: *Springer The Protein Protocols Handbooks*, 3rd edn (ed. J.M. Walker), Humana Press, Hatfield, UK, pp. 569–574.

86 Barbaglio, A., Tricarico, S., Ribeiro, A. et al. (2015) Ultrastructural and biochemical characterization of mechanically adaptable collagenous structures in the edible sea urchin *Paracentrotus lividus*. *Zoology.* 118 (3), 147–160.

87 Mredha, M., Zhang, X., Nonoyama, T. et al. (2015) Swim bladder collagen forms hydrogel with macroscopic superstructure by diffusion induced fast gelation. *Journal of Materials Chemisty B.* 3 (39), 7658–7666.

88 Piez, K.A. (1984) Molecular and aggregate structures of the collagens, in *Extracellular Matrix Biochemistry* (eds. K.A. Piez, A.H. Reddi). Elsevier Science Ltd, New York, 1984, pp. 1–40.

89 Mizuno, K., Hayashi, T., Peyton, D.H. et al. (2004) Hydroxylation-induced stabilization of the collagen triple helix. Acetyl-(glycyl-4(R)-hydroxyprolyl-4(R)-hydroxyprolyl) (10)-NH(2) forms a highly stable triple helix. *Journal of Biological Chemistry.* 279, 38072–38078.

90 Li, Y., Asadi, A., Monroe R.M. et al. (2009) pH effects on collagen fibrillogenesis in vitro: Electrostatic interactions and phosphate binding. *Materials Science and Engineering C.* 29 (5), 1643–1649.

91 Achilli, M., Mantovani, D. (2010) Tailoring mechanical properties of collagen-based scaffolds for vascular tissue engineering: the effects of pH, temperature and ionic strength on gelation. *Polymers.* 2 (4), 664–680.

92 Hoyer, B., Bernhardt, A., Lode, A. et al. (2014) Jellyfish collagen scaffolds for cartilage tissue engineering. *Acta Biomaterialia.* 10 (2), 883–892.

93 Bierbaum, S., Hintze, V., Scharnweber, D. (2012) Functionalization of biomaterial surfaces using artificial extracellular matrices. *Biomatter.* 2 (3), 132–141.

94 Ferrario C., Leggio, L., Leone, R. et al. (2016) Marine-derived collagen biomaterials from echinoderm connective tissues. *Marine Environmental Research.* 128, 46–57.

95 Mayne, J., Robinson, J.J. (2001) Comparative analysis of the structure and thermal stability of sea urchin peristome and rat tail tendon collagen. *Journal of Cellular Chemistry.* 84 (3), 567–574.

96 Leikina E., Mertts, M.V., Kuznetsova, N. et al. (2012) Type I collagen is thermally un- stable at body temperature. *Proceedings of the National Academy of Science of the United States of America.* 99, 1314–1318.

97 Lai, G., Li, Y., Li, G. (2008) Effect of concentration and temperature on the behavior of collagen solution. *International Journal of Biological Macromolecules.* 42 (3), 285–291.

98 Kao, N., Bhattacharya, N.S. (1998) The effect of temperature on the viscoelastic properties of model and industrial dispersions. *Journal of Rheology.* 42, 493–506.

99 Forgacs, G., Newman, S.A., Hinner, B. et al. (2003) Assembly of collagen matrices as a phase transition revealed by structural and rheologic studies. *Biophysical Journal.* 84 (2), 1272–1280.

100 Amis, E.J., Carriere, C.J., Ferry, J.D. (1985) Effect of pH on collagen flexibility determined from dilute solution viscoelastic measurements. *International*

Journal of Biological Macromolecules. 7 (3), 130–134.

101 Vader, D., Kabla, A., Weitz, D. et al. (2009) Strain-induced alignment in collagen gels. *PLoS ONE.* 4 (6), e5902.

102 Drzewiecki, K.E., Parmar, S.A., Gaudet, D.I. et al. (2014) Methacrylation induces rapid, temperature-dependent, reversible self-assembly of type-I collagen. *Langmuir.* 30 (37), 11204–11211.

103 Yoshimura, K., Chonan, Y., Shirai, K. (1999) Preparation and dynamic viscoelastic characterization of pepsin-solubilized collagen from shark skin compared with pig skin. *Animal Science Journal.* 70 (4), 227–234.

104 Zhang, M., Chen, Y, Li, G. et al. (2010) Rheological properties of fish skin collagen solution: Effects of temperature and concentration. *Korea-Australia Rheology Journal.* 22 (2), 119–127.

105 Carey, F.G; Teal, M.J. (1969) Regulation of body temperature by the bluefin tuna. *Comparative Biochemistry and Physiology.* 28 (1), 205–213.

106 Gudmundsson, M; Hafsteinsson, H. (1997) Gelatin from cod skins as affected by chemical treatments. *Journal of Food Science.* 62 (1), 37–39.

107 Montero, P., Gòmez-Guillén M.C. (2000) Extracting conditions for megrim (*Lepidorhombus boscii*) skin collagen affect functional properties of the resulting gelatin. *Journal of Food Science.* 65 (3), 434–438.

108 Liu, H.Y., Li, D., Guo, S.D. (2008) Extraction and properties of gelatin from channel catfish (*Letalurus puneatus*) skin. *LWT – Food Science and Technology.* 41 (3), 414–419.

109 Walters, B.D., Stegemann, J.P. (2014) Strategies for directing the structure and function of three-dimensional collagen biomaterials across length scales. *Acta Biomaterialia.* 10 (4), 1488–1501.

110 Lee P., Lin, R., Moon, J. et al. (2006) Microfluidic alignment of collagen fibers for in vitro cell culture. *Biomedical Microdevices.* 8 (1), 35–41.

111 Antman-Passig, M., Levy, S., Gartenberg, C. et al. (2017) Mechanically oriented 3D collagen hydrogel for directing neurite growth. *Tissue Engineering Part A.* 23 (9-10), 403–414.

112 Li, Y., Douglas, E.P. (2013) Effects of various salts on structural polymorphism of reconstituted type I collagen fibrils. *Colloids and Surfaces B: Biointerfaces.* 112, 42–50.

113 Park, S.N., Park, J.C., Kim, H.O. et al. (2002) Characterization of porous collagen/hyaluronic acid scaffold modified by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide cross-linking. *Biomaterials.* 23 (4), 1205–1212.

114 Safandowska, M., Pietrucha, K. (2013) Effect of fish collagen modification on its thermal and rheological properties. *International Journal of Biological Macromolecules.* 53, 32–37.

115 Fortunati, D., Chau, D.Y., Wang, Z. et al. (2014) Cross-linking of collagen I by tissue transglutaminase provides promising biomaterial for promoting bone healing. *Amino Acids.* 46 (7), 1751–1761.

116 Delgado L.M., Bayon, Y, Pandit, A. et al. (2015) To cross-link or not to cross-link? Cross-linking associated foreign body response of collagen-based devices. *Tissue Engineering Part B: Reviews.* 21 (3), 298–313.

117 Engler, A.J., Sen, S., Sweeney, H.L. et al. (2006) Matrix elasticity directs stem cell lineage specification. *Cell.* 126 (4), 677–689.

118 Hongwei, L., Lisha, L., Meiyu, S. et al. (2015) Mechanism of regulation of stem cell differentiation by matrix stiffness. *Stem Cell Research & Therapy.* 6 (1), 103.

119 Cozzolino, A.M., Noce, V., Battistelli, C. et al. (2016) Modulating the substrate stiffness to manipulate differentiation of resident liver stem cells and to improve the differentiation state of hepatocytes. *Stem Cells International*. 2016, ID 5481493.

120 Harley, B.A., Leung, J.H, Silva, E.C et al. (2007) Mechanical characterization of

collagen-glycosaminoglycan scaffolds. *Acta Biomaterialia.* 3 (4), 463–474.

121 Ng, K.W., Khor, H.L, Hutmacher, D.W. (2004) In vitro characterization of natural and synthetic dermal matrices cultured with human dermal fibroblasts. *Biomaterials.* 25 (14), 2807–2818.

122 Zidek, J., Vojtova, L., Abdel-Mohsen, A.M. et al. (2016) Accurate micro-computed tomography imaging of pore spaces in collagen-based scaffold. *Journal of Material Science: Materials in Medicine.* 27 (6), 110.

123 Reese, S.P., Farhang, N., Poulson, R. et al. (2016) Nanoscale imaging of collagen gels with focused ion beam milling and scanning electron microscopy. *Biophysical Journal.* 111 (8), 1797–1804.

124 Gaspar, A., Moldovan, L., Costantin, D. et al. (2011) Collagen-based scaffolds for skin tissue engineering. *Journal of Medicine and Life.* 4 (2), 172–177.

125 Taraballi, F., Corradetti, B., Minardi, S. et al. (2016) Biomimetic collagenous scaffold to tune inflammation by targeting macrophages. *Journal of Tissue Engineering.* 7, 2041731415624667.

126 Torbet, J., Ronzière M.C. (1984) Magnetic alignment of collagen during self-assembly. *Biochemical Journal.* 219 ((3), 1057–1059.

127 Hoyer, B., Bernhardt, A., Heinemann, S. et al. (2012) Biomimetically mineralized salmon collagen scaffolds for application in bone tissue engineering. *Biomacromolecules* 13 (4), 1059–1066.

128 Raftery, R.M., Woods, B., Marques, A.L. et al. (2016) Multifunctional biomaterials from the sea: Assessing the effects of chitosan incorporation into collagen scaffolds on mechanical and biological functionality. *Acta Biomaterialia* 43,160–169.

129 Elango, J., Zhang, J., Bao, B. et al. (2016) Rheological, biocompatibility and osteogenesis assessment of fish collagen scaffold for bone tissue engineering. *International Journal of Biological Macromolecules.* 91, 51–59.

130 Matsumoto, Y., Ikeda, K., Yamaya, Y. et al. (2011) The usefulness of the collagen and elastin sponge derived from salmon as an artificial dermis and scaffold for tissue engineering. *Biomedical Research.* 32 (1), 29–36.

131 Terada, M., Izumi, K., Ohnuki, H. et al. (2012) Construction and characterization of a tissue-engineered oral mucosa equivalent based on a chitosan-fish scale collagen composite. *Journal of Biomedical Materials Research Part B: Applied Biomaterials.* 100 (7), 1792–1802.

132 Jridi, M., Bardaa, S., Moalla, D. et al. (2015) Microstructure, rheological and wound healing properties of collagen-based gel from cuttlefish skin. *International Journal of Biological Macromolecules.* 77, 369–374.

133 Chandika, P., Ko, S.C., Oh, G.W. et al. (2015) Fish collagen/alginate/chitooligosaccharides integrated scaffold for skin tissue regeneration application. *International Journal of Biological Macromolecules.* 81, 504–513.

134 Nicklas, M., Schatton, W., Heinemann, S. et al. (2009) Preparation and characterization of marine sponge collagen nanoparticles and employment for the transdermal delivery of 17-beta-estradiol-hemihydrate. *Drug Development and Industrial Pharmacy.* 35 (9), 1035–1042.

135 Nicklas, M., Schatton, W., Heinemann, S. et al. (2009) Enteric coating derived from marine sponge collagen. *Drug Development and Industrial Pharmacy.* 35 (11), 1384–1388.

136 Swatschek, D., Schatton, W., Müller, W. et al. (2002) Microparticles derived from marine sponge collagen (SCMPs): preparation, characterization and suitability for dermal delivery of all-trans retinol. *European Journal of Pharmaceutics and Biopharmaceutics.* 54 (2), 125–133.

137 Ailen, A., Martinez-Alvarez, O. (2013) Marine collagen as a source of bioactive molecules: a review. *The Natural Products Journal.* 3 (2), 105–114.

138 Choi, S.S., Regenstein J.M. (2000) Physicochemical and sensory characteristics of fish gelatin. *Journal of Food Science.* 65 (2), 194–199.

139 Liu, C., Sun, J. (2015) Hydrolyzed tilapia fish collagen induces osteogenic differentiation of human periodontal ligament cells. *Biomedical Materials.* 10 (6), 065020.

140 Berthiaume, F., Maguire, T.J., Yarmush, M.L. (2011) Tissue engineering and regenerative medicine: history, progress, and challenges. *Annual Review of Chemical and Biomolecular Engineering.* 2, 403–430.

141 Baroli, B. (2009) From natural bone grafts to tissue engineering therapeutics: brainstorming on pharmaceutical formulative requirements and challenges. *Journal of Pharmaceutical Sciences.* 98 (4), 1317–1375.

142 Ozbolat, I.T, Hospodiuk, M. (2016) Current advances and future perspectives in extrusion-based bioprinting. *Biomaterials.* 76, 321–343.

143 Jose, R.R., Rodriguez, M.J., Dixon, T.A. et al. (2016) Evolution of bioinks and additive manufacturing technologies for 3D bioprinting. *ACS Biomaterials Science & Engineering.* 2 (10), 1662–1678.

144 Malda, J., Visser, J., Melchels, F.P. et al. (2013) 25th Anniversary Article: Engineering hydrogels for biofabrication. *Advanced Materials.* 25 (36), 5011–5028.

145 Mandrycky, C., Wang, Z., Kim, K. et al. (2016) 3D bioprinting for engineering complex tissues. *Biotechnology Advances.* 34 (4), 422–434.

146 Murphy, S.V., Atala, A. (2014) 3D bioprinting of tissues and organs. *Nature Biotechnology.* 32, 773–785.

147 Billiet, T., Gevaert, E., De Schryver, T. et al. (2014) The 3D printing of gelatin methacrylamide cell-laden tissue-engineered constructs with high cell viability. *Biomaterials.* 35 (1), 49–62.

148 Jakus, A.E., Rutz, A.L., Shah, R.N. (2016) Advancing the field of 3D biomaterial printing. *Biomedical Materials.* 11 (1), 014102.

149 Kang, H.W., Lee, S.J., Ko, I.K. et al. (2016) A 3D bioprinting system to produce human-scale tissue constructs with structural integrity. *Nature Biotechnology.* 34, 312–319.

150 Hutton, D.L, Grayson, W.L. (2014) Stem cell-based approaches to engineering vascularized bone. *Current Opinion in Chemical Engineering.* 3, 75–82.

151 Kolesky, D.B., Truby, R.L., Gladman, A. et al. (2014) 3D bioprinting of vascularized, heterogeneous cell-laden tissue constructs. *Advanced Materials.* 26 (19), 3124–3130.

152 Jungst, T., Smolan, W., Schacht, K. et al. (2015) Strategies and molecular design criteria for 3D printable hydrogels. *Chemical Reviews.* 116 (3), 1496–1539.

153 Fedorovich, N.E., Schuurman, W., Wijnberg, H.M. et al. (2012) Biofabrication of osteochondral tissue equivalents by printing topologically defined, cell-laden hydrogel scaffolds. *Tissue Engineering Part C: Methods.* 18 (1), 33–44.

154 Zhao, Y., Li, Y., Mao, S. et al. (2015) The influence of printing parameters on cell survival rate and printability in microextrusion-based 3D cell printing technology. *Biofabrication.* 7 (4), 045002.

155 Oliveira, S.M., Reis, R.L., Mano, J.F. (2015) Towards the design of 3D multiscale instructive tissue engineering constructs: Current approaches and trends. *Biotechnology Advances.* 33 (6), 842–855.

156 Lee, Y.B., Polio, S., Lee, W. et al. (2010) Bio-printing of collagen and VEGF-releasing fibrin gel scaffolds for neural stem cell culture. *Experimental Neurology.* 223 (2), 645–652.

157 Shin, K.H. Kim, J.-W., Koh, Y.H. et al. (2015) Novel self-assembly-induced 3D plotting for macro/nano-porous collagen scaffolds comprised of nanofibrous collagen filaments. *Materials Letters.* 143, 265–268.

158 Lode, A., Meyer, M., Brüggemeier, S. et al. (2016) Additive manufacturing of collagen scaffolds by three-dimensional plotting of highly viscous dispersions. *Biofabrication.* 8 (1), 015015.

159 Inzana, J.A., Olivera, D., Fuller, S.M. et al. (2014) 3D printing of composite calcium phosphate and collagen scaffolds for bone regeneration. *Biomaterials.* 35 (13), 4026–4034.

160 Lee, J.W., Choi, Y.J., Yong, W.J. et al. (2016) Development of a 3D cell printed construct considering angiogenesis for liver tissue engineering. *Biofabrication.* 8 (1), 015007.

161 Park, J.Y., Shim, J.-H. Choi, S.A. et al. (2015) 3D printing technology to control BMP-2 and VEGF delivery spatially and temporally to promote large-volume bone regeneration. *Journal of Materials Chemistry B.* 3, 5415–5425.

162 Shim, J.H., Jang, K.M., Hahn, S.K. et al. (2016) Three-dimensional bioprinting of multilayered constructs containing human mesenchymal stromal cells for osteochondral tissue regeneration in the rabbit knee joint. *Biofabrication.* 8 (1), 014102.

163 Wu, Z., Su, X., Xu, Y. et al. (2016) Bioprinting three-dimensional cell-laden tissue constructs with controllable degradation. *Scientific Reports.* 6, 24474.

164 Yeo, M., Lee, J.-S., Chun, W., et al. (2016) An innovative collagen-based cell-printing method for obtaining human adipose stem cell-laden structures consisting of core–sheath structures for tissue engineering. *Biomacromolecules.* 17 (4), 1365–1375.

165 Lee, H.J., Kim, Y.B. Ahn, S.H. et al. (2015) A new approach for fabricating collagen/ECM-based bioinks using preosteoblasts and human adipose stem cells. *Advanced Healthcare Materials.* 4 (9), 1359–1368.

166 Hinton, T.J., Jallerat, Q., Palchesko, R.N. et al. (2015) Three-dimensional printing of complex biological structures by freeform reversible embedding of suspended hydrogels. *Science Advances.* 1 (9), e1500758.

167 Rhee, S., Puetzer, J.L., Mason, B. et al. (2016) 3D Bioprinting of spatially heterogeneous collagen constructs for cartilage tissue engineering. *ACS Biomaterials Science & Engineering.* 2 (10), 1800–1805.

168 Wollensak, G., Spoerl, E., Seiler, T. (2013) Riboflavin/ultraviolet-a-induced collagen crosslinking for the treatment of keratoconus. *American Journal of Ophthalmology.* 135 (5), 620–627.

169 Trotter, J.A., Lyons-Levy, G., Luna, D. et al. (1996) Stiparin: a glycoprotein from sea cucumber dermis that aggregates collagen fibrils. *Matrix Biology.* 15 (2), 99–110.

170 Trotter, J.A., Lyons-Levy, G., Chino, K. et al. (1999) Collagen fibril aggregation-inhibitor from sea cucumber dermis. *Matrix Biology.* 18 (6), 569–578.

171 Tipper, J.P., Lyons-Levy, G., Atkinson, M.A. et al. (2002) Purification, characterization and cloning of tensilin, the collagen-fibril binding and tissue-stiffening factor from *Cucumaria frondosa* dermis. *Matrix Biology.* 21 (8), 625–635.

172 Yamada, A., Tamori, M., Iketani, T. et al. (2010) A novel stiffening factor inducing the stiffest state of holothurian catch connective tissue. *Journal of Experimental Biology.* 213 (Ptc20), 3416–3422.

173 Takehana, Y., Yamada, A., Tamori, M. et al. (2014) Softenin, a novel protein that softens the connective tissue of sea cucumbers through inhibiting interaction between collagen fibrils. *PLoS One.* 9 (1), e85644.