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Extraction Methods, Characterization and Biomedical Applications of Collagen: a Review

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Abstract: It is difficult to develop a standard extraction method for all types of collagen from different tissues due to the extreme diversity of collagen types. Some procedures are based on the isolation of acid, pepsin, and enzymatic soluble collagen, showing certain advantages and disadvantages. Other methods were also optimized to partially purify collagen and extract it easier than the methods currently used. Indeed, this review describes some advantages and disadvantages of these isolation methods. Moreover, major biomedical applications of collagen were reported. Given the great importance of biocompatible matrices in tissue engineering, the availability of native collagen should be investigated by refining the collagen extraction procedure.

Keywords: collagen; purification; tissue engineering; biomedical applications.

1. Introduction

Collagen is the major component of the extracellular matrix. It is a fibrillar protein composing different conjunctive tissue forms such as bone, cartilage, tendon, and skin [1-4]. Collagen can form insoluble fibrils with high resistance characteristics and can induce or regulate many structural and cellular functions and processes such as differentiation, movement, communication, and apoptosis [5- 7].

Type I, II, and III collagens are the most abundant and well investigated for biomedical applications as a plastic material in medicine and cosmetology, but also in the pharmaceutical industry as compounds that prolong the action of drugs [5, 8, 9], as well as a natural scaffold in tissue engineering and reconstructive medicine (especially type I) [10].

There are almost 20 different types of collagen in humans, each encoded by a specific gene. However, the various types of collagens have slightly different amino acid compositions and perform specific body functions. The main types of collagen are type I (all tissues and

organs), type II (exclusive to cartilage), type III (skin, blood vessels, and organs), type IV (basement membranes as a system of filtration), and type V (all tissues as a cytoskeleton).

These functions are due to the properties of collagen as a protein. The emphasis on type I collagen is due to its ability to form fibrils with a length of 300 nm and a fibrillar diameter of up to 1000 nm. This collagen type is trimeric [(α1)2β2] and naturally exists as a triple helix. These helices have "Gly-X-Y" repeats (where X and Y mainly Pro and Hyp). Thus, proline and hydroxyproline, commonly known as imino acids, constitute about 23% of the total protein sequence, and the Gly-Pro-Hyp structure is the most common form often based [11].

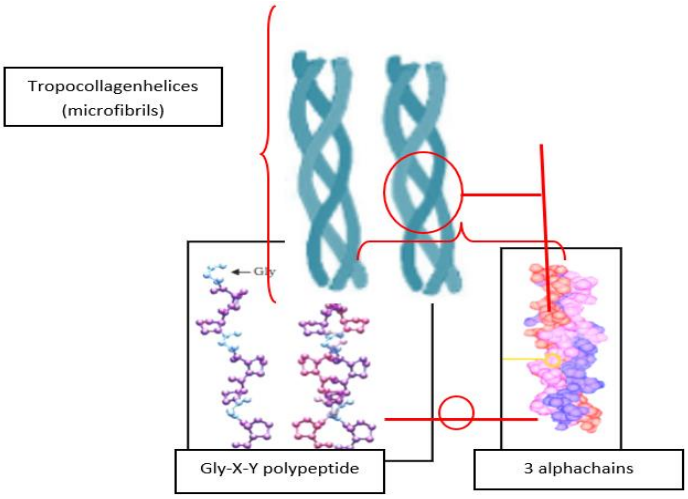

Figure 1. Structure of collagen.

Beyond this extreme diversity of tissues and collagen types, it is important to develop a standard extraction method for all types of collagen and its different sources. The increase over time of the number of covalent intermolecular interactions in collagen structure frequently results in almost complete insolubility in the solvents used for proteins [5, 6, 12]. This work's main objective was to research, study and analyze the known methods of collagen isolation and purification to evaluate their efficiency and optimize them at minimum conditions and normal necessities. The implication for health policy/practice/research/medical education: Physicochemical characterization, as well as all biomedical applications of collagen from different sources, were discussed.

2. Collagen Isolation and Characterization

The various types of collagen can be isolated from different sources. In general, the materials are suspended in cold distilled water for 2-3 days, with water changing twice a day. Then the materials are cut into small pieces (about 1 cm in length).

To isolate the collagen, several methods have been used, such as extraction with neutral salt, acid, and enzymatic solutions [6, 13-18]. Indeed, the salt precipitation method was one of the salt extraction procedures based on treating pieces with neutral salt solutions. Then the collagen is isolated by gradually increasing the sodium chloride concentration (adding NaCl). The supernatant, containing the salt-soluble collagen fraction, could be dialyzed [14]. At the same time, the acid isolation method is based on extracting collagen from pieces with an organic acid. The supernatant, which contained an acid-soluble fraction of collagen, could be as in the salt method [5, 6]. The enzymatic isolation method is based on the extraction of collagen with an organic acid in the presence of pepsin. The supernatants of the solutions

extracted are salted out with NaCl, and the precipitate obtained (content in pure collagen) is dissolved in acetic acid. Then the solution was dialyzed against Na_2HPO_4. The general scheme of collagen isolation and purification is shown in figure 1.

After having tested several collagen isolation procedures, it was noted that the efficiency of the basic salt extraction is low. Also, the solubilization capacity could be obtained by increasing the salt concentration, which will increase the ionic power of the solution obtained. However, in normal tissues, the proportion of neutral salt soluble collagen is usually unimportant, so the final yield is very low.

The alternative method used to ensure the extraction is based on solubilization with dilute organic acid acetic or citric acid used as a solvent the acetic acid in the presence of EDTA, which effectively inhibits tissue degradation. Clearly, in comparison, this method has a greater ability to solubilize collagen than neutral salt extraction but is still limited to young uncrosslinked collagen.

The acid extraction method generally presents a high yield, which was not the case for some studies [19, 20], due to the source (scales) of the first study and the minimum conditions and the simplicity of the materials used in the second. Also, the yield of ASC obtained by [19] was 0.37% (dry weight basis), which was similar to that of ASC from spotted golden goatfish scales (0.46%) [21] but significantly lower than that of ASC from carp scales (0.86%) [22]. Moreover, Kim and Park [23] mentioned the effectiveness of the pepsin method (+ 34% compared to ASC). The ASC yield was also low for [24] (0.58% on a dry weight basis), and it was greyish, while the yield of PSC was comparatively higher (16.23% on a dry weight basis), pinkish, and fiber-like.

The low levels of collagen content may be due to the proteins' denaturation during the process and the difference in ambient temperature [25].

Nagai [26] found less yield of ASC than PSC from diamondback squid (*Thysanoteuthis rhombus*) skin, which was about 1.3% on a dry weight basis. On the other hand, the PSC was perfectly solubilized, and its yield was very high, about 35.6% on a dry weight basis. Additionally, Shanmugam *et al*. [24] extracted collagen (expressed in dry weight) from the dried skin of *Sepiellainermis*. However, the collagen content in many animals, on a wet weight basis, reported higher values. Moreover, the pepsin method's yield depends on the concentration of acetic acid used in this manipulation, as demonstrated in the study by Kiew and Mat Don [27] between two different concentrations of 0.7 M and 0.9 M, which recorded 26.69 and 20.35 on average, respectively.

On the other hand, the amino acid compositions per 1000 in total showed variation in the composition of collagens. Indeed, the residues of acid-soluble collagen (ASC) and pepsin soluble collagen (PSC) were rich in proline (Pro), glycine (Gly), and hydroxyproline (Hyp), which were due to characteristic $(Gly\text{-}Pro\text{-}Hyp)_n$, a triple-helical repeat of all collagens. High levels of alanine (Ala), as observed in the collagens of animal species, have also been measured in the fish scale collagens [28].

In addition to Ala, a high level of Hyp was detected, and low levels of His, Hyl and Tyr were generally observed with some cases of Trp absence (case noted in the study of [19]. Except for Cys-s, the other amino acid compositions of these acid-soluble and pepsin-soluble collagen sources were similar to those of ordinary muscle type I collagen [29] and dermal collagen porcine type I [30]. The freshwater fish scales contained relatively high Cys-s, while there were almost no Cys-s detected in other seawater fish collagens [31-35]. The degrees of

hydroxylation of proline generally varied between extraction methods, which would eventually affect the stability of collagen fibers and denaturation temperatures [30, 33].

The results obtained by analyzing these amino acid determination studies indicated that the experiment of removing non-collagenous proteins was appropriate. Gly is considered the most dominant amino acid in collagen, as known in all members of the collagen family; the results showed the domains with repeats of the proline-rich tripeptides (Gly-XY) involved in the formation of the triple helix, except for the first 14 amino acid residues from the N-terminus and the first 10 amino acid residues from the C-terminus of collagen molecules, where X is usually Pro and Y is mainly Hyp. On the other hand, the Gly content of ASC-C (347.1 residues/1000 residues) was higher than that (328–341 residues/1000 residues) of ASC from carp scales [36], deep-sea redfish [37], sardine, red sea bream, Japanese sea bass [38], and spotted golden goatfish [39], but lower than those of ASC from rohu (361 residues/1000 residues) and catla (353 residues/1000 residues) scales [40]. Additionally, the amounts of imino acids (Pro and Hyp) are important for collagen's structural integrity. In particular, Hyp is believed to have a major role in stabilizing the triple-stranded collagen helix due to its ability to hydrogen bond through its hydroxyl group. Therefore, the ASC-C helices could be more or less stable depending on the number of residues due to the imino acid content (higher/lower) which the pyrrolidine rings (Pro and Hyp) imposed restrictions on the conformation of the polypeptide chain and helped to enhance the thermal stability of the triple helix [41].

Regarding the denaturation temperature (DT) of ASC from multipurpose sources, it was largely higher than the Td of PSC, mainly due to enzymatic hydrolysis. The DT of the ASC of the used fish scale sources was lower than that of porcine skin collagen but higher than the Td of many cold-water fish collagens measured under the same conditions [42, 39, 43]. Previous studies showed that the stability of collagen is correlated with environmental and body temperatures [44], but current studies have shown that hydroxyproline is important for maintaining the stability of trimmers in collagen [30, 33].

The different Tmax of transitions among collagen from animal species seems to be correlated with the content of imino acids (proline and hydroxyproline). The higher the imino acid content, the more stable the helices [45]. Moreover, the stability of collagen is correlated with room and body temperature [44]. The collagen of cold-water fish has a low imino acid content [46]. Also, the increase in the content of imino acids (proline and hydroxyproline) led to an increase in the DT of collagen [47-49].

In contrast, acetic acid has played an essential role in changing collagen's thermal properties, especially in the skin. In fact, the skin and bone collagens rehydrated in acetic acid exhibited decreases in Tmax and transition enthalpy (DH) compared to those rehydrated in water.

Acetic acid can cleave hydrogen bonds [50], which stabilizes collagen in a triple-helical structure [51]. Thus, the collagen structure has been disrupted, resulting in a decrease in collagens' thermal stability, as shown by the decrease in Tmax and enthalpy. Other studies demonstrated that a DT variation may be due to the type, sex, or age of the resources used [20].

3. Biomedical Applications

Collagen is very abundant in nature in various forms and having several properties necessary for different applications in biomedical sciences. Indeed, it is a versatile biomaterial with wide medical applicability.

3.1. Tissue regeneration.

Tissue regeneration or regenerative medicine is the set of techniques using engineering, cell culture, life sciences, and materials sciences to develop biological substitutes that can restore, maintain, or improve tissue functions. It is often based on the use of a scaffold that will serve as a support for the growth of new viable tissue.

3.1.1. Oral mucosa tissue regeneration.

Various types of natural and synthetic biomaterials have been used to engineer oral mucosa, including collagen hydrogel from rat-tails and chitosan-fish scale collagen (Table 1). Using primary oral keratinocytes, Terada *et al*. [52] reported that a chitosan-collagen composite scaffold was constructed by blending commercial chitosan and tilapia scale collagen multilayered, polarized, and stratified epithelial layer with superficial keratinization. In another study, Tabatabaei *et al*. [53] investigated the viability of collagen hydrogel in the oral mucosa tissue engineering using human primary oral fibroblast and keratinocyte cells that isolated from gingival biopsies. The results showed the seeded keratinocytes' adherence onto the fibroblast-populated collagen gel and development of a multilayered stratified epithelium on its surface after three days of cultivation, collagen hydrogels encapsulating fibroblasts increased cell viability.

Table 1. Oral mucosa tissue regeneration.

Form	Origin	Extraction technique	Biological evaluation	Results	References
Scaffold	Tilapia fish scales	Freeze-drying Dehydrothermal cross-linked	Primary oral keratinocytes	Produced multilayered, polarized, stratified epithelial layer with superficial keratinization	[52]
Collagen hydrogel	Rat tails	Freeze-drying	Human primary oral broblast and keratinocyte cells	Increased cell viability Formation of a stratified and differentiated epithelium on the surface of cell-laden collagen hydrogel	[53]
Collagen Peptides (MCPs)	Tilapia skin		The tongue mucosa of C57/BL6 mice	Effectively accelerated the healing process of oral ulcer	[54]
collagen film			*In vivo* on 36 male chinchilla rabbits	The decrease of clinical signs of inflammation Faster and massive growth of soft tissue	[55]

3.1.2. Vascular tissue regeneration.

Several approaches have reported that collagen has been used as a biomaterial in various vascular tissue applications because of its excellent biocompatibility (Table 2). Jeong *et al*. [56] studied the feasibility of jellyfish (*Stomolophus nomurim eleagris*) collagen as tissue-engineered vascular grafts in pulsatile perfusion bioreactor using vascular smooth muscle cells (SMCs) and endothelial cells (ECs). As a result, it was shown that the co-culturing of SMCs and ECs on collagen/PLGA hybrid scaffolds under a pulsatile perfusion system induced the cellular alignment, the enhancement of vascular EC development, and the retention of differentiated cell phenotype. This study also demonstrated that the jellyfish collagen/PLGA scaffolds up-regulated smooth muscle expressions and endothelial cell activity-related molecules. On the other hand, using mouse lymphatic endothelial cell line, fish scale-derived collagen showed a favorable integration to the surrounding tissues, with good infiltration of

cells, blood vessels (BVs), and lymphatic vessels (LVs), as well as improved cell attachment and proliferation [57].

Table 2. Vascular tissue regeneration.

Form	Origin	Extraction technique	Biological evaluation	Results	References
Scaffold	Jellyfish (*Stomolophus nomurim eleagris*)	Electrospinning	Smooth muscle cells Endothelial cells	Enhanced cell proliferation Induced cell alignment Up-regulated expressions smooth muscle and endothelial cell activity-related molecules Enhanced endothelial cell development	[56]
Scaffold	Snakehead scales	Freeze-drying 1,4-butanediol diglycidyl ether (BDE) cross-linked	Mouse lymphatic endothelial cell line	Improved cell attachment, proliferation and infiltration Favorable growth of blood and lymphatic vessels	[57]

3.1.3. Skin tissue and wound healing.

Until today, numerous natural and synthetic collagen-based biomaterials have received great attention due to their beneficial biological functions on skin tissue and wound healing [58-80] (Table 3). Indeed, Zhang *et al*. [59] investigated the wound healing potential of administering marine collagen peptides (MCP) from Chum Salmon (*Oncorhyn chusketa*) skin using two wound models (incision and excision) *in vivo*. As a result, it was shown that MCP increased wound closure and improved tissue regeneration at the wound site and improved angiogenesis and helped form thicker and better-organized collagen fiber deposition. The MCP also increased the formation of a capillary, fibroblast, and collagen fiber, the expression of platelet-endothelial cell adhesion molecule-1, basic fibroblast growth factor, and TGF-B1 in rats following cesarean section [62]. Furthermore, the scaffold that was prepared by low-molecular-weight fish scale collagen peptides (FSCP) and chitooligosaccharides (COS) showed good biocompatibility *in vitro* and supported the proliferation of human skin fibroblasts [58]. In another work, the fish collagen/alginate (FCA) sponge scaffold improved cell adhesion and proliferation and exhibited the best cellular compatibility in human dermal cells [61]. Vigneswari *et al*. [63] demonstrated that P(3HB-co-4HB)/FSCP (fish-scale collagen peptides) scaffolds provided better cell attachment and growth of L929 mouse fibroblast cells and better cell proliferation as well as accelerated wound contractions. Using human fibroblasts and keratinocytes, mrigal fish (*Cirrhinus cirrhosus*) scale scaffolds enhanced cell growth, attachment, and proliferation and increased wound healing rate, re-epithelialization, and dermal reconstitution [64]. Moreover, Zhou *et al*. [69] demonstrated that fish collagen, obtained from tilapia skin, promoted the adhesion, proliferation, and migration of human keratinocytes. This collagen also induced the secretion of type one collagen and vascular endothelial growth factor by human dermal fibroblasts, further stimulating the proliferation of human vascular endothelial cells and accelerated rat skin wound healing.

Also, Zhang *et al*. [68] used mouse fibroblasts (NIH-3T3) to evaluate the mechanical properties and the biocompatibility of pepsin-soluble collagen isolated from the skin of *Leiocassislongirostris* by uniaxial tensile mechanical testing and cell proliferation assay, respectively. In this study, the collagen revealed a denser network structure with thicker fibrils and better uniaxial tensile mechanical properties and could provide a much more suitable environment for cell growth and migration.

Additionally, Pozzolini *et al*. [71] studied wound-healing of marine collagen hydrolysates (MCHs)-from the marine sponge *C. reniformis* using fibroblasts keratinocytes, and the survival of both cells was evaluated after UV radiation. The results showed that MCH demonstrated promising wound-healing properties, facilitating both cell migration and proliferation at the site of the wound of epidermal and dermal cells. In another study, the various composition chitosan/fish collagen/glycerin 3D porous scaffolds were fabricated *via* freeze-drying technique and investigated their effect on mechanical strength, biostability, and cytocompatibility *in vitro* culture of human fibroblasts and keratinocytes. This study showed the good cytocompatibility of scaffolds and excellently facilitated cell proliferation and adhesion [70]. Using the rat wound model, Chen *et al*. [72] revealed that four types of collagen, including pepsin soluble collagen sponge (PCS), acid-soluble collagen sponge (ACS), bovine collagen electrospun I (BCE I), and bovine collagen electrospun II (BCE II) increased the percentage of wound contraction, reduced the inflammatory infiltration, and accelerated the epithelization and healing. Another work carried out by Wang *et al*. [73] collagen matrix produced by SCCO 2 technology revealed chemically similar to human skin type I collagen, non-toxic, good biocompatibility, and accelerated wound healing in porcine excision full-thickness skin wound model.

Recently, Ge *et al*. [79] evaluated the collagen chitosan scaffold's effect alone or enriched with either bone marrow-derived mesenchymal stem cells (BM-MSCs) or their secreted extracellular vesicles (EVs) on the duration and quality of skin wound healing *in vivo*. The results showed that collagen chitosan scaffolds significantly accelerated the rate of skin healing, enhanced macrophages, and increased collagen deposition. Also, Ge *et al*. [79] showed that collagen hydrogel fabricated by freeze-drying for Nile tilapia skin (*Oreochromis niloticus*) accelerated the healing of deep second-degree burns wounds and promoted the formation of new skin appendages. On the other hand, using hamster fibroblasts (V79) native collagen extracted from adult *paracentrotuslividus* byenzymatical hydrolysis method increased cell proliferation, reduced water evaporation, and protein diffusion, as well as acting as a barrier against bacterial infiltration [78].

Table 3. Skin tissue and Wound healing.

Form	Origin	Extraction technique	Biological evaluation	Results	References
Scaffold	Fish scale collagen peptides chito-oligosaccharides	Electrospinning	Human skin fibroblasts	Good biocompatibility *in vitro* Supported fibroblast proliferation	[58]
Collagen peptide	Chum salmon (*Oncorhynchusketa*) skin	Enzymatical hydrolysis	Rat wound model (incision and excision) *in vivo*	Accelerated the wound closure Improved tissue regeneration at the wound site Improved angiogenesis Increased organized collagen fiber deposition	[59]
Scaffold	Fish scale collagen *Macrotylomauniflorum* extracts	Freeze-drying Cross-linked with glutaraldehyde	NIH-3T3 HaCaT	Good biocompatibility with both cell lines	[60]
Scaffold	Flatfish (*Paralichthysolivaceus*) skin	Freeze-drying EDC cross-linked	Human dermal cells	Induced cell adhesion and proliferation	[61]

Form	Origin	Extraction technique	Biological evaluation	Results	References
				Promoted well-spread cell morphology	
Collagen peptide	Chum salmon (*Oncorhynchusketa*) skin	Enzymatical hydrolysis	Rat wound model (following cesarean section) *in vivo*	Accelerated the wound healing process in rats	[62]
Scaffold	*Tilapia* fish skin	Cross-linked with glutaraldehyde	L929 mouse fibroblast cells Rat wound model *in vivo*	Enhanced cell growth, attachment, and proliferation Accelerated wound contractions	[63]
Scaffold	Mrigal fish (*Cirrhinuscirrhosus*) scale	Freeze-drying Cross-linked with glutaraldehyde	Human fibroblasts and keratinocytes Rat wound model *in vivo*	Enhanced cell growth, attachment, and proliferation Increased wound healing rate, re-epithelialization, and dermal reconstitution	[64]
Scaffold	Fish scale collagen	Freeze-drying Cross-linked with ceftazidime	NIH-3T3 fibroblast cell line	Good biocompatibility	[65]
Scaffold	Weever skin	Freeze-drying EDC/NHScross-linked	Mouse embryonic fibroblasts cells Rabbit wound model *in vivo*	Promoted biocompatibility Increased cell growth and proliferation Reduced inflammation Enhanced tissue regeneration and healing	[66]
Collagen peptide	Nile tilapia (*Oreochromisniloticus*)skin	Enzymatical hydrolysis	Human keratinocyte Rabbit scald wound model *in vivo*	Increased cell proliferation Promoted wound healing	[67]
Scaffold	*Leiocassislongirostris*skin	EDC cross-linked	Mouse fibroblasts (NIH-3T3)	Facilitated cell proliferation and migration	[68]
Scaffold	Tilapia skin	Electrospinning	Human keratinocytes (HaCaT) Human dermal fibroblasts (HDFs) Rat skin defect model *in vivo*	Promoted the adhesion, proliferation, and migration of HaCaT Induced the secretion of type I collagen and vascular endothelial growth factor by HDFs Accelerated rat skin wound healing	[69]
Scaffold	*Tilapia* fish scale	Freeze-drying	Human keratinocytes and fibroblasts	Good cytocompatibility Facilitated cell proliferation and adhesion	[70]
Collagen peptide	*Chondrosiareniformis*	Enzymatical hydrolysis	Mouse macrophage cell line Mouse fibroblast L929 cell line HaCaT	Increased cell proliferation Induced a photo-protective effect	[71]
Scaffold	Tilapia skin	Freeze-drying	Rat wound model *in vivo*	Increased wound contraction	[72]

Form	Origin	Extraction technique	Biological evaluation	Results	References
				Reduced inflammatory reaction Enhanced collagen synthesis and dermal reconstitution Accelerated the epithelization and wound healing	
Collagen matrix	Porcine skin	Freeze-drying	Fibroblastsandkeratinocytes Pig wound model *in vivo*	No toxic effect Excellent biocompatibility (*in vivo* et *in vitro*) Decreased inflammation, completed epithelization, and enhanced wound healing (*in vitro*)	[73]
Collagen hydrogel	Porcine skin	Freeze-drying	Human epidermal keratinocytes	Induced fast and superior skin regeneration in a non-healing wound model in diabetic mice	[74]
Collagen-chitosan membranes	Porcine skin	Cross-linked with alginate dialdehyde	L929 fibroblasts cells Rat skin resection wound	No cytotoxicity toward L929 fibroblasts Good biocompatibility Promoted wound healing in vivo	[75]
Scaffold	Skin of newborn lambs	Freeze-drying	Rat wound model *in vivo* Bone marrow-derived mesenchymal stem cells	Improved wound healing	[76]
Scaffold	Bovine tendons	Freeze-drying	Bone mesenchymal stem cells Rat wound model *in vivo*	Increased cell adhesion, viability, and differentiation Improved wound healing (*in vivo*)	[77]
Native collagen	Adult *Paracentrotuslividus*	Enzymatical hydrolysis	Hamster fibroblasts (V79)	Increased cell proliferation Reduced water evaporation and protein diffusion Acted as a barrier against bacterial infiltration	[78]
Collagen hydrogel	Nile Tilapia Skin (*Oreochromisniloticus*)	Freeze-drying	NIH-3T3 fibroblast cell line Rats' skin-deep second-degree burns	No significant toxicity to fibroblasts Accelerated the healing of deep second-degree burn wounds Promoted the formation of new skin appendages	[79]

3.1.4. Bone tissue regeneration.

Bone tissue is constantly changing; this process gives a bone the self-healing properties. However, in some cases, this natural self-repair process is insufficient because of mechanical or biological problems. Therefore, bone reconstruction must be assisted; this is the stake in the bioengineering of bone. In fact, biomaterial-based bone grafts have an important role in the

field of bone tissue engineering. In this context, several studies show that collagen, particularly the collagens of marine origin, has interesting osteoconductive and biomechanical properties and is applied increasingly in tissue engineering [81-99] (Table 4).

Pallela *et al*. [96] evaluated the scaffold (Chi-HAp-MSCol) derived from *Thunnus obesus* bone and marine sponge (*Irciniafusca*) collagen (MSCol) on MG-63 cell line *in vivo*. This scaffold was prepared using the freeze-drying and lyophilization method. According to this study, these biomimetic scaffolds have potential in the field of bone tissue engineering [96]. Also, the collagen extracted from the freshwater fish origin, using a technique of extraction called Freeze-drying Cross-linking using 1-ethyl-3- (3-dimethyl-aminopropyl) -carbodiimide (EDC), was evaluated for its biocompatibility and immunogenicity *in vitro* on fibroblasts (3T3) cells and human osteosarcoma cells (MG63), and *in vivo* using the mouse model. The results revealed a significant proliferation rate of cells on the scaffolds, and in 5 days, the cells were fully confluent [96]. The addition of Aquamin to the collagen–GAG biomaterial has improved osteoblasts' mineralization and enhanced osteogenesis to facilitate bone repair *in vivo* [90]. Moreover, Xu *et al*. [91] showed that the marine collagen peptides (MCP) derived from chum salmon (*Oncorhyn chusketa*) skin on the development of femurs in growing rats; the result showed that the MCP increases the size, mineral density, dry weight, ash weight, most mineral content and both stiffness and toughness of the femurs in growing male rats [91]. In another study, Mredha *et al*. [88] developed a novel class of collagen fibril-based tough hydrogels based on the double network (DN) collagen (SBC), extracted from Bester sturgeon fish. The implantation of the gels in the rabbit knee's osteochondral defect showed that these DN hydrogels exhibit excellent biomechanical performance in vivo and have a strong bonding ability with bone [88].

The evaluation of physicochemical and morphological characteristics, as well as biological performance in vitro of the association of HA (hydroxyapatite) and SPG (called spongin) composites, showed that this combination improves the biological properties, in particular those mimicking bone composition (with 70% HA and 30% SPG) [86]. In another study, [74] found that the biphasic scaffold develops from marine collagens are a suitable setup for *in vitro* chondrogenic and osteogenic differentiation of human mesenchymal stromal cells (hMSC). Also, Nabavi *et al*. [83] prepared collagen-based hydrogel scaffolds containing tacrolimus and surrounded by a PCL/gelatin membrane; the results provide evidence of the developed efficacy hydrogel for the treatment of bone defects. In vitro and in vivo biological assessments of collagen scaffolds, fabricated via the SSM model, improved osteogenesis of rBMSCs and modulated the macrophage response, thus positively affecting bone regeneration [82].

Tsai *et al*. [81] used HANF fragments to evaluate the effects of COL-HANF scaffolds on MG63 osteoblast-like cell behaviors, *in vitro* and *in vivo* properties showed that COL-HANF scaffold has potential as a bone graft for bone tissue engineering; in addition, the scaffolds have promoted the differentiation of MG63 osteoblast-like cells. Furthermore, Matsumoto *et al*. [98] studied Tilapia scale collagen fibril's effect on the differentiation of human mesenchymal stem cells (hMSCs). According to this study, the hMSCs adhered easily to tilapia scale collagen, which accelerated the early stage of osteoblastic differentiation in hMSCs *in vitro* cell culture. In another study, Hu *et al*. [67] investigated the therapeutic effects of collagenous peptides extracted from scales of two kinds of fish on the Human MG-63 osteosarcoma cell line. The results indicated that collagenous peptides promoted the

proliferation of osteoblasts; therefore, could be used to prevent osteoporosis from assisting bone remodeling [67].

Table 4. Bone tissue regeneration.

Form	Origin	Extraction technique	Biological evaluation	Results	References
Collagen peptide	Growing rat femora model (*in vivo*)	Enzymatical hydrolysis	Chum salmon (*Oncorhyn chusketa*) skin	Enhanced stiffness and toughness of femurs Increased size, weight, and mineral density and content of femurs	[91]
Insoluble	Purchased	EDC and NHScross-linked	Human bone marrow cells	Incorporated on the surface of the porous hydroxyapatite scaffolds	[97]
Scaffold	*Thunnusobesus* bone and marine sponge (*Irciniafusca*) collagen	Freeze-drying and lyophilization method	Human MG-63 osteosarcoma cell line	Promoted cell proliferation	[96]
Scaffold	Fish scales of Rohu and Catla	Freeze-drying Cross-linking using 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide (EDC)	Fibroblasts (3T3) cells Human osteosarcoma cells (MG63) Mouse model (*in vivo*)	Elicited minimal inflammatory response Promoted cell proliferation	[95]
Collagen peptide	Bone and skin from cods	Enzymatical hydrolysis	Human osteoblasts	Promoted cell proliferation Up-regulated the expression of the osteogenic markers Accelerated the matrix mineralization	[94]
Collagen peptide	Gadiformes and Pleuronectidae	Enzymatical hydrolysis	Mouse calvaria-derived MC3T3-E1 cells	Up-regulated the expression of collagen modifying enzymes Increased collagen deposition Accelerated the matrix mineralization	[93]
Collagen peptide	Scale of tilapia	Enzymatical hydrolysis	Rat bone marrow-derived mesenchymal stem cells	Promoted cell viability Up-regulated the expression of the osteogenic markers Up-regulated the expression of the endothelial marker	[92]
Native collagen	Tilapia scale collagen	Freeze-drying	Human mesenchymal stem cells (hMSCs)	Accelerated the early stage of osteoblastic differentiation Up-regulated the osteoblastic markers	[98]
Scaffold	Glycosaminoglycan Aquamin	Freeze-drying Dehydrothermal (DHT) cross-linking treatment	Mouse pre-osteoblast MC3T3-E1 cell line	Improved mineralization	[90]
Collagen peptide	Sparidae and Chanos	Enzymatical hydrolysis	Human MG-63 osteosarcoma cell line	Promoted the proliferation of osteoblasts Inhibited the proliferation of mature osteoclasts	[67]
Scaffold	Blue shark (*Prionaceglauca*) cartilage	Freeze-drying Glutaraldehyde cross-linked	Human acute T-lymphocyte leukemia cell lines (6T-CEM) Human fetal osteoblasts	Increased cell viability Enhanced the alkaline phosphatase activity	[100]

Scaffold	Rat-tail tendon	Neutralization	Mesenchymal dental pulp stem cell simplanted in a rat critical-sized calvarial defect model	Restored the osteoblastic forming and osteoclastic resorbing processes	[89]
Scaffold	Swim bladder of Bester sturgeon fish	Glutaraldehyde and genipin cross-linked	Rabbit bone defect model *in vivo*	Good biomechanical performance Strong bonding ability with bone	[88]
Scaffold	Sharkskin (*Prionaceglauca*) collagen Shark teeth bioapatite	Freeze-drying Cross-linking using EDC/NHS (N-Hydroxysuccinimide) and hexamethylene diisocyanate (HMDI)	Human osteoblast-like cell line (Saos-2)	Increased cell viability	[87]
Scaffold	Marine sponge *Aplysinafulva*	Vacuum drying	Mouse fibroblasts (L929) Mouse pre-osteoblastic cells (MC3T3-E1)	Promoted cell viability	[86]
Scaffold	Biomimetically mineralized salmon collagen and fibrillated jellyfish collagen	Freeze-drying EDC cross-linked	Bone marrow-derived human mesenchymal stromal cells	Induced chondrogenic and osteogenic differentiation	[85]
Scaffold	Fish scale and skin	Electrospinning	Bone mesenchymal stem cells Human gingiva fibroblasts cells	Improved cytocompatibility Enhanced the mechanical strength and accelerated the degradation rate	[84]
Scaffold	Rat-tail tendon	EDC cross-linked	Human osteosarcoma cell line Rat calvarias defect model*in vivo*	Promoted *in vitro* cell proliferation Induced the bone healing (*in vivo*)	[83]
Scaffold	Rat (Sprague-Dawley) tail tendon fascicles	Lyophilization EDC and NHScross-linked	Rat bone marrow mesenchymal stromal cells	Promoted *in vitro* cell proliferation and osteogenic differentiation Improved osteogenesis by altering the macrophage response	[82]
Scaffold	Calfskin	Freeze-drying EDC cross-linked	MG63 osteoblast-like cell Rabbit condylar defect model *in vivo*	Promotedcell proliferation Promoted bone regeneration	[81]
Scaffold	Human-like collagen (HLC)		MC3T3-E1 osteoblast cells	Excellent mechanical and superior biological properties for bone tissue regeneration	[101]

3.1.5. Cartilage tissue regeneration.

Deformities or damage to the cartilaginous facial structures (nose or auricle) can occur due to trauma, tumor resection, or congenital disabilities. Reconstruction of these defects requires intervention using autologous grafts such as a rib or ear cartilage or synthetic materials such as Gore-Tex or silicone. Nevertheless, there is a need for new cartilage replacement strategies, such as tissue engineering, which is based on the use of autologous chondrocytes and resorbable matrices (Table 5). To this end, a study conducted by Bermueller *et al*. [102] demonstrated that marine collagen matrices offer excellent properties for cartilage tissue engineering, using marine collagen scaffolds, thus preventing septal perforation in an autologous and orthotopic rat model. In this sense, and during this study, it was possible on the

one hand to study the relevance of marine collagen as a replacement matrix for cartilage in the context of three-dimensional cultures *in vitro* by analyzing cell migration, cytotoxicity, and extracellular matrix formation using human and rat nasal septal chondrocytes.

On the other hand, the researchers proceeded to develop an orthotopic animal model suitable for the repair of the nasal septum while at the same time evaluating the biocompatibility of marine collagen. For this purpose, histological and immunohistochemical evaluation of seeded and unseeded scaffolds transplanted into nasal septum defects in an orthotopic rat model (for 1, 4, and 12 weeks) showed that the scaffolds did not induce any cytotoxic reactions *in vitro*. The chondrocytes were able to adhere to marine collagen and produce cartilage matrix proteins, such as type II collagen. Likewise, treatment of septal cartilage defects *in vivo* with seeded and unseeded scaffolds resulted in a significant reduction in the number of nasal septal perforations compared to no replacement. This novel experimental surgical procedure provides a suitable means to evaluate new scaffolding materials for their applicability in repairing nasal cartilage. In another research work carried out by (Ohnishi *et al*. [103] on 12 rabbits, in which osteoarthritis was induced, the researchers studied the correlations of the severity of osteoarthritis (OA) and serum biomarkers extracted from the collagen of Gadiformes fish species, including the epitope of keratan sulfate, hyaluronic acid, and chondroitin sulfate 846. Likewise, during this study, the effect of glucosamine and collagen peptide extracted from fish on osteoarthritis was also investigated. Osteoarthritis was induced in 12 rabbits (12 weeks old) by anterior cruciate ligament transection. After the surgery, the rabbits were orally administered fish collagen peptide (group F), glucosamine (group G) or fish collagen peptide, and glucosamine (group FG) for 4 weeks. The control group received water ad libitum (group C). Also, to measure serum markers, blood samples were taken before surgery (pre-transaction of the anterior cruciate ligament) and before euthanasia (post-transection of the anterior cruciate ligament).

During this study, a macroscopic and histological assessment of the severity of osteoarthritis was performed. The results showed that the condylar surfaces were slightly eroded in group C. Additionally, the histological results were significantly different from those of the FG group and the other groups. There were no significant differences between each group during anterior cruciate ligament transection in terms of serum keratan sulfate, hyaluronic acid, and chondroitin sulfate 846.

Histological evaluation and serum biomarker measurements performed after anterior cruciate ligament transection showed a significant correlation between hyaluronic acid concentration and the severity of osteoarthritis. Changes in chondroitin sulfate 846 concentration at the pre-anterior cruciate ligament and post-anterior cruciate ligament transection levels were significantly correlated with osteoarthritis severity.

Administration of glucosamine and fish collagen peptide had chondroprotective effects in the anterior cruciate ligament transection model. Serum biomarker concentrations were significantly correlated with cartilage damage. Measurement of serum biomarkers would be useful for monitoring articular cartilage damage in clinical settings.

Tilapia Fish collagen may provide a suitable collagen source for chondrogenesis of human mesenchymal stem cells (hMSCs) *in vitro*; a novel alternative to conventional mammalian collagens such as bovine and porcine collagen. In another study conducted by Hsu *et al*. [104], researchers investigated the chondrogenic differentiation of hMSCs grown on tilapia-scale collagen fibrils compared to porcine collagen uncoated dishes. This research study showed that fish collagen could overcome zoonosis risk, like that of bovine spongiform

encephalopathy. In particular, tilapia collagen, whose denaturation temperature is close to 37°C, was ideal for cell and tissue culture. In this study, the scanning electron microscope was used to observe collagen fibrils. Safranin O staining, expression of glycosaminoglycans (GAGs), showed that hMSCs cultured on collagen at the tilapia scale exhibited stronger safranin O staining and higher expression of GAGs on day 6.

Real-time PCR was also used to assess the chondrogenesis of hMSCs on each type of collagen fibrils showing that hMSCs has grown on tilapia collagen exhibited earlier expression of SOX9 on day 4 with higher expression of AGGRECAN and COLLAGEN II on day 6 compared to porcine collagen and uncoated dishes. Furthermore, low bone gammacarboxyglutamate mRNA levels, a specific osteogenesis marker, showed that tilapia collagen fibrils specifically enhance chondrogenic differentiation of hMSCs in chondrogenic media, as well as porcine collagen. Therefore, tilapia scale collagen can provide a suitable collagen source for chondrogenesis of hMSCs cultured *in vitro*. In the same context of using collagen extracted from fish, marine hybrid constructions of porous scaffolds from fibrillated jellyfish collagen and alginate hydrogel mimic the two main components' cartilage tissue. This constitutes a promising approach for the chondrogenic differentiation of hMSCs. Indeed, a study by Pustlauk *et al*. [105] showed the potential of hybrid scaffolds based on marine biomaterials-hydrogel alginate in repairing articular cartilage, and this by examining scaffolds either infiltrated with an alginate cell suspension or seeded with hMSCs and incorporated in the alginate after cell adhesion. The researchers also compared the hybrid constructs with 2 x 105 and 4.5 x 105 hMSCs/scaffold and hMSCs encapsulated in pure alginate discs, both chondrogenically stimulated for 21 days. They revealed a typical round chondrocyte-like morphology in pure alginate gels and alginate-cell-suspension scaffolds, while the cells of the scaffolds embedded in the alginate after seeding had an elongated shape and were tightly attached to the collagen pores. However, the Col 2/Col 1 ratio was higher for pure alginate discs and alginate cell suspension scaffolds than for scaffolds embedded in alginate after seeding. Compared to the porous hydrogel-free jellyfish collagen scaffolds, hMSCs embedded in hybrid scaffolds showed higher gene expression of chondrogenic markers. Additionally, the secretion of sulfated glycosaminoglycans was comparable for the alginate cell suspension scaffolds and the scaffolds incorporated into the alginate after seeding. Finally, the results of this study showed that hybrid collagen and alginate constructs from jellyfish support chondrogenic differentiation of hMSCs and provide more stable constructs compared to pure hydrogels. Accordingly, another study performed by Diogo *et al*. [106], whose findings were consistent with the previous study, highlighted the relevance of using blue shark collagen biopolymer as a building block to produce highly efficient temporary matrices in cartilage applications. This *in vitro* study revealed that human adipose-derived stem cells (hASCs) adhere abundantly to constructs, thus promoting early chondrogenic differentiation of these cells. This work addressed the potential of 3D collagen-based structures of blue shark (*Prionaceglauca*) skin to promote the differentiation of hASCs into a chondrogenic lineage with and without exogenous stimulation. The cryogelation method was applied using a mixture with hyaluronic acid to enhance the constructs' microporous interconnectivity [107]. The interconnected microporous structures have been shown to promote cell adhesion and cell proliferation and extracellular matrix (ECM) formation and infiltration in scaffolds, showing great potential for regeneration of cartilage tissue, especially when considering the use of cell-free strategies. The results obtained confirm *P. glauca* cutaneous collagen's biomedical applicability, representing a strategy for enhancing marine by-products.

The potential of blue shark (*P. glauca*) skin collagen to induce chondrogenic differentiation of hASCs was investigated, with and without exogenous stimulation. For this purpose, a cryogelation method has been applied to produce highly interconnected porous three-dimensional constructs based on collagen and hyaluronic acid. These results are supported by the expression of markers encoding chondrogenesis-related mRNA (Coll II and Sox-9), which are strongly up-regulated at an early stage for both conditions, with or without exogenous stimulation. This assumes that *P. glauca* collagen itself can support chondrogenic differentiation at early times, but exogenous stimulation is required to ensure the phenotype's maintenance. Likewise, Raabe *et al*. [108] evaluated the potential of hydrolyzed fish collagen in the differentiation and chondrogenesis of stromal cells derived from equine adipose tissue, and therefore, the application of stromal cells derived from adipose tissue in equine veterinary tissue engineering, especially for repairing cartilage. To do this, this work focused on the study of the effect of transforming growth factor beta1 (TGF-B1) compared to hydrolyzed fish collagen in terms of the chondrogenic differentiation potential of stromal cells derived from adipose tissue, knowing that these cells are multipotent cells which, in the presence of appropriate stimuli, can differentiate into various lineages such as osteogenic, adipogenic, and chondrogenic cells. In this study, stromal cells derived from adipose tissue were isolated from horses' subcutaneous fat by liposuction. Chondrogenesis was studied using a pellet culture system. The differentiation medium was either supplemented with the growth factor of TGF-B1 (5 mg/mL) or fish collagen (0.5 mg/mL) during 3 weeks of differentiation *in vitro*. Moreover, examining the degree of chondrogenic differentiation and the formation of the cartilage extracellular matrix was carried out using the RT-PCR technique and histological staining to synthesize proteoglycans and type II collagen.

The differentiation of adipose tissue-derived stromal cells induced by TGF-B1 showed a high expression of GAG. Histological analysis of cultures stimulated with hydrolyzed fish collagen demonstrated even higher GAG expression than cultures stimulated under standard conditions with TGF-B1.

The expression of cartilage-specific type II collagen and Sox9 was approximately the same in the two stimulated cultures. In this study, chondrogenesis was induced as efficiently by hydrolyzed fish collagen as by TGF-B1. The results demonstrated that hydrolyzed fish collagen alone can induce and maintain chondrogenesis derived from stromal cells derived from adipose tissue.

Many approaches in this field have shown partially satisfactory results. Cartilage tissue engineering, combining innovative scaffolds and stem cells from different sources, appears to be a promising strategy for cartilage regeneration. To this end, another recent study conducted in rats by Szychlinska *et al*. [109] aimed to assess the ability of a type I collagen scaffold to promote cartilage repair after orthotopic implantation *in vivo*.

Articular cartilage lesions were created at the patellofemoral groove in rat knees with type I collagen scaffolds implantation. At 4, 8, and 16 weeks after transplantation, degrees of cartilage repair was assessed by morphological, histochemical, and gene expression analyzes. This study's conclusions are in line with previous studies [102-103], which suggested that the collagen-based scaffold, especially type I, is highly biocompatible and able to recruit host cells from surrounding joint tissue to promote cartilage repair of joint defects. In a recent study, led by Zhang *et al*. [110], authors developed an injectable hydrogel system composed of collagen I-tyramine and hyaluronic acid-tyramine and built bone marrow mesenchymal stem cells and a hydrogel-charged system for cartilage regeneration. The first results showed that this

injectable hydrogel could be used ideally in the regeneration of cartilage tissues. Then, this hydrogel system's physicochemical properties were well characterized and optimized, in particular the gel time, the rigidity, the water absorption, and degradability. In this study, an evaluation of the proliferation and differentiation of mesenchymal stem cells of the bone marrow in a hydrogel composed of type I collagen-hyaluronic acid was performed. An examination of the repair capacity of cartilage *in vivo* in the presence of TGF-B1 was also performed. The results of this research suggest a wide range of collagen applications in the biomedical field. This hydrogel system showed high biocompatibility, supported the chondrogenic differentiation of mesenchymal stem cells in the bone marrow, and allowed appropriate hyaline cartilage repair.

Table 5. Cartilage tissue regeneration.

Form	Origin	Extraction technique	Biological evaluation	Results	References
Collagen peptide	Skin of deepwater ocean fish (cod, haddock, and pollock)	Enzymatic hydrolysis	Horse adipose-derived stromal cells	Increased glycosaminoglycan expression Induced chondrogenic differentiation	[108]
Collagen peptide	Skins of Gadiformes species	Enzymatic hydrolysis	Rabbit osteoarthritis model *in vivo*	Chondroprotective effects	[103]
Scaffold	Jellyfish *Rhopilemae sculentum*	Freeze-drying EDC cross-linked	Primary human and rat nasal septum chondrocytes Rat septal cartilage defect model *in vivo*	Promoted adhesion and cartilaginous matrix proteins production Reduced nasal septum perforations	[102]
Collagen solution	Tilapia fish scale collagen	Acid soluble collagen isolation method	Human mesenchymal stem cells	Enhanced chondrogenic differentiation Elevated expression of chondrogenic markers Increased glycosaminoglycan expression	[104]
Scaffold	Jellyfish *Rhopilemae sculentum*	Freeze-drying EDC cross-linked	Primary human mesenchymal stem cells	Induced chondrogenic differentiation	[105]
Cryogel	Blue shark (*Prionaceglauca*) skin	Cryogelation EDC cross-linked	Human adipose stem cells	Promoted chondrogenic cell differentiation	[106]
Scaffold	Not reported	Freeze-drying	Articular cartilage lesion orthotopic model	Induced the formation of fibrous tissue Replaced the scaffold with the newly formed cartilage-like tissue Displayed the expression of typical cartilage markers	[109]
Hydrogel	Not reported	Freeze-drying EDC and NHS cross-linked	Rat bone marrow mesenchymal stem cell Cartilage defect model *in vivo*	Promoted chondrogenic cell differentiation with a great biocompatibility Induced good hyaline cartilage repair	[110]

3.1.6. Corneal tissue regeneration.

The healthy cornea is a tough, transparent anterior surface of the eye that is essential for visual acuity. Corneal damage is a major cause of vision disturbances leading to limbal stem cell deficiency. To this end, several therapeutic strategies are being developed to treat limbal stem cell deficiency (Table 6). Indeed, Krishnanet and colleagues developed (*in vitro*) a novel source of collagen from fish scales (*Latescalcarifer*) to develop the biocompatible

scaffold for culturing limbal stem cells, which perfectly replace the human amniotic membrane [36]. An evaluation of fish scales' physicochemical, mechanical, and cultural characteristics was compared to the denuded human amniotic membrane. Furthermore, cultured corneal cells were characterized by an RT-PCR for putative stem cell markers. This study showed that collagen's mechanical and physical forces derived from fish scales were good enough to be manipulated relative to the human amniotic membrane. Observation under a light microscope showed epithelial migration after 48 h from limbal explants plated on collagen isolated from fish scales and on the human amniotic membrane after 72 h. At the end of the 15th day, confluent growth comparable to the morphological characteristics of the limbal epithelium was recorded. Also, the collagenase assay, assessment of swelling rate, and microbial resistance of fish scales gave better results than the human amniotic membrane. In summary, this study has shown that collagen derived from fish scales (*L. calcarifer*) can be used as a new material in corneal tissue engineering. Another study in rat models demonstrated the fish scale-derived collagen matrix's potential as an alternative for human donor corneal tissue [111]. Indeed, several parameters were measured, namely the diffusion and the transmission of the light from the collagen matrix derived from fish scales and their comparison with those of the cornea human. The short-term biocompatibility of this collagen matrix was also tested in this *in vivo* model.

The measurement of light scattering was performed using a stray light meter, and light transmission was measured using a broadband absorption spectrometer.

To examine biocompatibility, three methods were adopted; implantation of the collagen matrix derived from fish scales in anterior lamellar keratoplasty, its placement in an interlamellar and subconjunctival corneal pocket.

Transparency, neovascularization, and epithelial lesions were monitored for 21 days. Cell morphology and infiltration were evaluated histologically.

The preliminary results demonstrated that the amount of light scattered was comparable to that seen in early cataracts. The percentage of light transmission was similar to the transmission through the human cornea. The results of light scattering and transmission data showed that the early version of this fish scale-derived collagen matrix was comparable to human corneal tissue in this regard.

Histological examination showed chronic inflammation varying from mild to moderate in the anterior lamellar keratoplasty group and the interlamellar corneal pocket to severe in the subconjunctival group. Despite the technical difficulties, it was possible to use the collagen matrix derived from fish scales for anterior lamellar keratoplasty, while the placement of an interlamellar corneal pocket led to the fusion of the anterior lamella. Therefore, further studies are needed to understand its immunogenicity better.

Table 6. Corneal tissue regeneration.

Form	Origin	Extraction technique	Biological evaluation	Results	References
Native collagen	Fish (Latescalcarifer)scales	Drying at 25°C	Primary human limbal epithelial cells	Enhanced cell viability, growth, proliferation, and migration Recorded a good swelling ratio and microbial resistance	[36]
Scaffold	Tilapia fish scale–derived collagen matrix	Decellularization Decalcification	Rat ocular implantation model *in vivo*	Biocompatible Adequate light transmission Reasonable light-scattering values	[111]

3.1.7. Dental tissue regeneration.

It has been reported that collagen has also proven a key role in dental tissue regeneration (Table 7). Indeed, different forms of collagen extracted by several techniques of extractions have shown their capacity to induce dental tissue regeneration, and therefore they can be used in biomedical applications to regenerate dental tissue [112-118]. Collagen peptide extracted from *Tilapia scale* using enzymatical hydrolysis was tested Rat odontoblast-like cell line [112] and human periodontal ligament cells [113]. The results showed that collagen improves cellular attachment and viability by up-regulation of gene expression and accelerating matrix mineralization [112]. Collagen also promoted cell viability and up-regulated gene expression of proteins of human periodontal ligament cells [113]. Moreover, collagen peptide extracted from Tilapia fish skin collagen type I by Freeze-drying showed an important capacity to enhance the level of osteocalcin secretion towards osteogenic differentiation in human periodontal ligament fibroblasts [115]. It was recently reported that collagen gel extracted from the dermis of market-weight pigs by polymerization exhibits a positive effect on dental tissue regeneration by secretion growth factors, promoting cell differentiation, and induced endothelial and osteogenic cell proliferation [116].

Table 7. Dental tissue regeneration.

Form	Origin	Extraction technique	Biological evaluation	Results	References
Collagen peptide	Tilapia scale derived type I collagen	Enzymatical hydrolysis	Rat odontoblast-like cell line	Increased initial cell attachment and cell viability Up-regulated osteogenic gene expression Accelerated matrix mineralization	[112]
Collagen peptide	Tilapia scales	Enzymatical hydrolysis	Human periodontal ligament cells	Promoted cell viability Up-regulated the expression of osteogenic markers Up-regulated the production of osteogenic-related proteins	[113]
Scaffold	Tilapia fish collagen	Elecrospinning	Human periodontal ligament cells A dog class II furcation defect model *in vivo*	Enhanced cell viability and osteogenic gene expression Promoted the expression of RUNX-2 and OPN protein Promoted bone regeneration	[114]
Collagen peptide	Tilapia fish skin collagen type I	Freeze-drying	Human periodontal ligament fibroblasts	Enhanced the level of osteocalcin secretion towards osteogenic differentiation	[115]
Collagen gel	Dermis of market-weight pigs	Polymerization	Dental pulp stem cells	Released growth factors Promoted cell differentiation Induced endothelial and osteogenic differentiation	[116]

3.1.8. Other applications.

Fibroblasts allow collagen to have dual functionality by ensuring the cohesion of tissues and organs *via* elastin and glycoproteins. On the other hand, by giving these tissues properties of flexibility, hydration, and resistance. In addition to the regeneration of the various tissues mentioned above, these properties have enabled collagen to be used in other applications.

Indeed, marine collagen has also been used in the food and agricultural industry by Prashant *et al*. [119] where they isolated and characterized acid-soluble collagen (ASC) from carp (*Cyprinuscarpio*) scales. Initially, the researchers aimed to produce paneer (a dairy product) by incorporating the extracted collagen. In the second part of the experiment, they

tested the ability of metabolites released (enzymatically treated) from the scales to promote root growth and seed germination of *Vignaradiata*. Therefore, the prepared paneer showed high acceptability with good textural and sensorial characteristics. Also, the metabolites released promoted plant growth, allowing them to be used as a nitrogen fertilizer source for plants.

Moreover, three-dimensional (3D) cell culture systems constitute a fundamental means for several biomedical studies (*in vitro* and clinical). Additionally, scaffolding presents a major tool in cell support (biocompatibility and mechanical strength) for 3D cell culture and tissue engineering. In this regard, Choi and colleagues fabricated a novel nanofiber scaffold composed of a mixture of fish scale collagen and polycaprolactone using the electrospinning technique [61].

These scaffolds were characterized by analyzing fiber diameter distribution and their biocompatibility using mouse thymic epithelial cells *via* the evaluation of several parameters such as adhesion, proliferation, spreading, and gene/protein expression. Therefore, the fabricated scaffolds favored these parameters by stimulating the expression of genes involved in cell adhesion and thymopoiesis molecules. Thus, these scaffolds can be an ideal model of 3D cell culture.

Otherwise, collagen can act as a hemostatic agent in the case of massive uncontrolled hemorrhages. The body's natural mechanism is not always able to control abundant hemorrhages. Thereby, several studies have proven the effectiveness of collagen in regulating hemostasis[120, 56] through the formation of platelet aggregates [121], the release of clotting factors, and the stimulation of platelets. In 2017, Cheng and collaborators extracted collagen from the jellyfish species *Rhopilema esculentum*, subsequently preparing a collagen sponge whose hemostatic ability was assessed by rat-tail amputation and whole-blood clotting experiments [122]. Consequently, the collagen sponges activated the hemostatic mechanism through physical absorption. Also, their application to experimental models of amputated rat-tails experiment reduced hemostasis time and bleeding; this may be attributed to the high rate of water absorption and the porous structure of collagen sponge.

Furthermore, myocardial tissue engineering constitutes a major tool for restoring and improving the functions of a diseased myocardium, hence developing suitable biomaterials for scaffolds supporting cardiomyocytes' properties. For this reason, several studies have chosen to evaluate natural polymers (collagen, gelatin, chitosan...). However, collagen application alone may present certain limitations for the scaffold (poor mechanical properties and accelerated degradation). In order to overcome these limitations, Fang *et al*. [123] incorporated chitosan with collagen for myocardial tissue engineering. For *in vivo* implantation, this collagen/chitosan composite scaffold requires stabilization by cross-linking to increase its strength further. Indeed, the authors comparatively studied three chemical cross-linking agents, namelygenip in (GP), glutaraldehyde (GTA), and tripolyphosphate (TPP). They found, as a result, high porosity (> 65%) for all scaffold groups with excellent mechanical properties for TPP cross-linked scaffolds. In addition, TPP and GP cross-linked scaffolds recorded the best biocompatibility compared to GTA cross-linked scaffolds. Also, cardiomyocytes in TPP cross-linked scaffolds showed the best contractile performance. This indicates that TPP presents the most suitable cross-linking agent for collagen/chitosan scaffolding in myocardial tissue engineering.

Regarding vocal fold tissue engineering, Walimbe *et al*. [124] evaluated four types of hydrogels (having tunable viscoelastic characteristics), incorporated with fibrillar type I collagen and type III collagen, for their biocompatibility, bioavailability, and influence on

vocal fold fibroblasts. Effectively, collagen's incorporation has significantly increased the mechanical properties of hydrogels, with better cell fixation and adhesion, creating a tissue microenvironment leading to remodeling.

Moreover, developing a novel source of collagen material has always been a major challenge in medical tissue engineering [125-127]. Indeed, Li *et al*. [128] chose aquatic collagen as an alternative to mammalian collagen. They isolated ASC and PSC (pepsin-soluble collagen) from the skin of tilapia (*Oreochromisniloticus*) in order to investigate their physicochemical and structural properties. Both collagens were characterized as type I collagen. In addition, they constructed tilapia skin collagen scaffolds that were implanted beneath the dorsal tissue of mice for tissue regeneration, using bovine collagen scaffolds as a control. As a result, the grafted scaffolds completely degraded without any inflammatory reaction, indicating their high biocompatibility and stability *in vivo*.

4. Conclusions and Perspectives

Collagen is a key substance for cells living and plays an important role in cellular and tissular homeostasis. In this review, it was reported that several natural resources could contain this substance, which possesses numerous physicochemical characteristics depending on the type of collagen. Importantly, several biomedical investigations showed that this molecule exhibits important pharmacological effects, particularly on the regeneration of different tissue. Therefore, this molecule can be introduced to medical benefit after further pharmacodynamic and pharmacokinetic investigations for its validation.

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Conflicts of Interest

The authors declare no conflict of interest.

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