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Review: fish collagen: extraction, characterization and application in wound healing and drug delivery

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Abstract. Collagen is a natural protein present in the animal and human body. It is in natural fibrous and can be found in the connective tissue and flesh of mammals, approximately 25 - 35 wt.% of total human protein. Collagen is abundant in fibrous tissues such as bones, cartilage, tendons, blood vessels, ligaments, skin, cornea, aortic disc, and intestines. Fish collagen is currently being studied as a new biological substitute for collagen from animals. It is extracted from by-products (scales, skins, bones, etc.) by biochemical or biological methods is safe, has high adsorption, high compatible. The factors influencing on the extraction process include temperature, time, concentration of ingredients, etc. The fish collagen is usually collagen type I with a fibril structure. Each unit of collagen is tropocollagen consisting of three polypeptide chains twisted together. Depending on the source of ingredients, the composition of amino acids, denaturation temperature, the sequence of amino acids, structure, morphology, molecular weight, extraction yield, etc. of obtained collagens are different. This paper reviews the extraction and characterization of fish collagen from products after fish processing by chemical or biochemical methods. The applications of fish collagen in wound healing and drug delivery are also reviewed.

Keywords: Chemical method, natural protein, collagen, drug delivery, extraction, biochemical method, wound healing.

Classification numbers: 2.3.1, 2.9.3, 2.10.2.

1. INTRODUCTION

Collagen possesses a triple helix structure, which is generally made up of two homologous chains (α-1) and one supplementary chain that varies slightly in its chemical composition (α-2). These chains are polypeptides in nature and coiled around one another in a cable form. Each has a distinct turn in the reverse direction, these chains are connected chiefly by hydrogen bonds between nearby CO and NH groups [1 - 4]. Collagen's structure unit is tropocollagen with a

length of 300 nm and a width of 1.5 nm [1, 2]. The tropocollagen can link together to form a microfibril structure. The microfibril then combines with each other to form bundle fibers and networks found in tissues, bones, and basal membranes. Each polypeptide chain contains about 1050 amino acids, mainly glycine (about 33 %), proline (about 25 %), and hydroxyproline. The amino acid sequence in each of three chains of collagen molecule is regularly arranged [2, 4]. The sequence of amino acids follows the pattern glycine-proline-X or glycine-X-hydroxyproline where X is other amino acids in collagen except glycine, proline, or hydroxyproline. Glycine constitutes about 1/3 of the total sequence and proline or hydroxyproline accounts for 1/6 of the sequence [3 - 5]. Intermolecular bonds of polypeptide chains in collagen are stabilized by hydrogen bonds between –CO– and –NH– groups on the polypeptide chains. The molecular weight of collagen is about 300 kDa [3]. The chemical structure of collagen type I is described in Figure 1 and the triple-helix structure of collagen is shown in Figure 2.

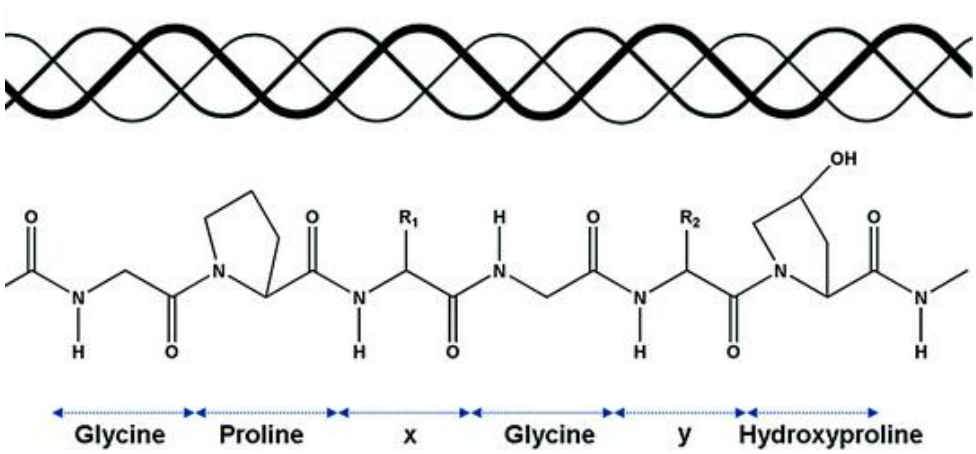

Figure 1. Chemical structure of collagen type I. Adapted from [3].

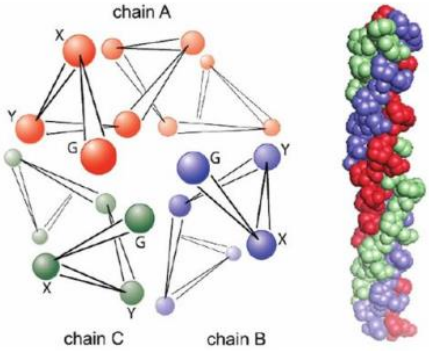

Figure 2. Triple-helix structure of collagen. Adapted from [2].

The amino acid composition of collagen differs from other proteins. Especially, collagen contains much glycine, proline, alanine, hydroxyproline, less hydroxylysine, and rarely cysteine and tryptophan. The hydroxyproline content could be used to determine the collagen content. The content of proline and hydroxyproline varies by species and their living environment conditions [6 - 10].

Collagen, with about 28 types, can be divided into two main groups: fibrous collagen and non-fibrous collagen including membrane collagen, granular collagen, short-chain collagen, transmembrane collagen, and some other types of collagens [2, 9].

Collagen can be dissolved in glycerol, acetic acid, and urea but it is insoluble in cold water or ethanol. It is lightly expandable, has good tensile strength, and is found both outside and inside the body cells. Besides, collagen is also highly biodegradable and biocompatible. When heated, collagen is easily converted into gelatin [11 - 12]. The number of acidic amino acids is balanced to the number of base amino acids; thus, the collagen retains its electrical equilibrium under different physiological conditions. It is swelled in water and reacts with acid or base [13].

Compared with other natural polymers, collagen is one of the best biological materials in the pharmaceutical industry such as capsules, hemostatic healing and wound healing, temporary blood substitution agent, tissue, and cell culture. In addition, using collagen to produce anti - biotic or anti - cancer drugs, tumor treatment is focused on the study [12 - 18].

Recently, fish collagen has attracted much attention in study and application in various fields such as biomedicine, healthy foods, cosmetics, etc. due to its high safety (no infectious diseases of land animal collagen), highly absorbable and biocompatible, degradable, no religious hindrances and cheap [12, 19 - 22]. Fish collagen is considered as a promising feedstock to replace land animal collagen. It has been used as a carrier for drug delivery systems [17, 18, 21, 23], in hemostatic membranes [24 - 27], in skin wound healing [28], in tissue regeneration [21, 29], in bone tissue engineering [30].

This article is to review about extraction, and characterization of fish collagen and apply it in drug delivery, wound healing and update the new results of fish collagen. Based on that, it can suggest the research directions in the coming time related to fish collagen and its applications.

2. METHODS FOR EXTRACTION OF FISH COLLAGEN

To obtain high-quality collagen, the parameters for extraction of fish collagen are very important. They consist of extraction method, extraction temperature, extraction time, source of fish by-products, ratio of solid/liquid, concentration of chemicals used in the extraction process, and so on. The by - products after fish processing can include skins, scales, bones, fins, blood, heads, intestines, gills, and fats. Among them, fish - skin, scale, fin, and bone by - products (Figure 3) have been used popularly for extraction of fish collagen [6, 31].

Some methods used for extracting collagen from fish by-products are the chemical method using acid or alkali [32 - 36], the biochemical method using enzyme or enzyme and acid or microorganisms and acid [37 - 40], extrusion-hydro-extraction [41], etc. Nevertheless, the extraction of collagen dissolved in acid (acid-soluble collagen) and in the presence of pepsin (pepsin-solubilized collagen) is widespread types. The extraction process of fish collagen is divided to 3 mainly stages: (i) cell breakdown, (ii) collagen extraction, and (iii) collagen purification. Collagen cannot move through cell membranes; thus, it is necessary to break down cell structure to transfer collagen into the medium. In this stage, the mill process of homogenization or organic solvents combined with cleaning agents can be applied to breakdown cell structure. This stage consists of the washing/cutting process and the pretreatment step. After the cell structure was broken, collagen was extracted using acids or enzymes. This stage was usually carried out at a low temperature. The mixture was also stirred continuously to enhance the contact of solid and solvent as well as to improve collagen quality. After this stage, collagen was precipitated and obtained raw, collagen in this form can be mixed with impure proteins,

lipids, mineral salts, etc. To remove these components, the dialysis or centrifugation method could be used to purify collagen. Finally, lyophilization is usually carried out to dry collagen. Figure 4 presents a general procedure for the extraction of collagen from fish by-products.

Figure 3. Several examples of fish by - products: (a) Fish scales, (b) fish fins, (c) fish skins, and (d) fish bones.

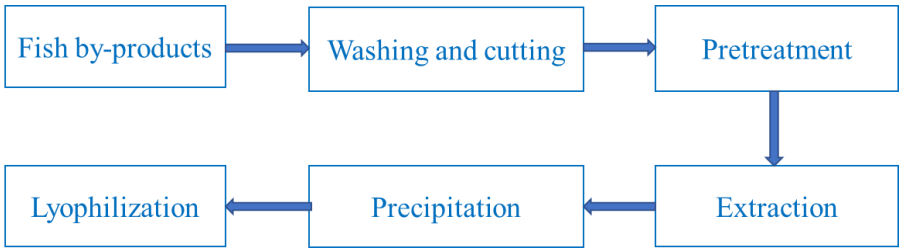

Figure 4. General procedure for extraction of collagen from fish by-products.

2.1. Chemical method for fish collagen extraction

The chemical method for fish collagen extraction is the extraction in acid or alkali conditions. The collagen obtained by using this method is often type I collagen with a fibril structure of triple helix chains. It is difficult to dissolve in water. It is used to be named acid soluble collagen (ASC). The time for extraction is quite long, from several days to weeks. The alkaline pretreatment is mostly applied in the first step of the extraction process. Sodium hydroxide or calcium hydroxide could be used; however, sodium hydroxide is more convenient

due to its better clean ability. The acidic solution is also used for demineralization from fish by-products in the case of scale, fin, cartilage, or born kinds. Lin Wang *et al.* studied the extraction of collagen from skins, scales, and bones of redfish (*Sebastes mentella*) [6]. The extraction process was carried out through the steps of reducing non-collagenous proteins with 1M NaCl solution; demineralized with 0.5 M ethylenediaminetetraacetic acid (EDTA) solution and defatted with n-hexane. These processes were performed for 24 hours before soaking obtained solid parts in 0.5 M acetic acid solution for 48 hours to extract and precipitate collagen with NaCl solution. The recovery efficiency and denaturation temperature of collagen from skin, scale, and bone were 47.5 %, 6.8 %, 10.3 %, and 16.7 ^{o}C, 17.7 ^{o}C, 17.5 ^{o}C, respectively. Muyonga *et al.* washed the skins of young and adult Nile perch (*Lates niloticus*) with NaCl 0.8 M before extraction of collagen by using acetic acid 0.5 M (solid/liquid of 1/20 (g/ml)). The time of extraction is 16 hours, the temperature of extraction is 15 ^{o}C. During extraction, an intermittent stirrer was applied. The yield of extraction for young fish skins and for adult fish skins was 63.1 % and 58.7 %, respectively [7]. Takeshi Nagai and Nobutaka Suzuki extracted collagen from the skins, bones, and fins of various fish species by chemical method [31]. The extraction procedures for all skins, bones, or fins were carried out at 4 ^{o}C. To remove non-collagenous proteins, the skins, bones, or fins were soaked with 0.1 N NaOH solution before washing with distilled water and lyophilizing. To obtain skin collagen, the non-collagenous protein skin was removed fat with 10 % butyl alcohol for 24 hours and then washed with distilled water and lyophilized. The solid part was immersed in 0.5 M acetic acid solution for 72 hours before centrifuging the extract at 20.000 × g for 1 hour. The residue was then re - extracted in the same solution for 48 hours and treated in the same conditions. Next, the solution part obtained after centrifuging was added with NaCl solution to precipitate collagen at near neutral pH (7.5). The precipitate was separated by centrifuging at 20.000 × g for 1 hour, and dissolved in 0.5 M acetic acid solution, then dialyzed in 0.1 M acetic acid solution, distilled water, and lyophilized after that. For bone collagen extraction, the insoluble bone was decalcified with 0.5 M EDTA solution (pH 7.4) for 5 days, the EDTA solution was changed once a day. Fat was removed with 10 % butyl alcohol after the residue was washed with distilled water. Fengxiang *et al.* extracted collagen type I from fresh carp fish scales by using NaCl solution to remove fats and proteins at the first step and using HCl 0.4 M solution to demineralize at the second step and using an acetic acid solution to extract collagen at the third step [32]. Dasong Liu *et al.* studied the influence of alkaline concentration in pretreatments and acid extraction conditions for extracting acid-soluble collagen (ASC) from grass carp (*Ctenopharyngodon idella*) skin [33]. The suitable concentration of NaOH solution is 0.05 - 0.1 M for pretreatment of carp skin to limit the loss of ASC. The concentration of the acetic acid solution is of 0.5 M to extract ASC completely. The processing temperature can be adjusted with the season instead of the commonly used 4 ^{o}C. Collagen was also extracted from fresh carp fish scales (*Cyprinus carpio*) by chemical method [10, 34]. The different alkali solutions ($Ca(OH)_2$ or NaOH or the mixture of $Ca(OH)_2$/NaOH) with various concentrations have been used for the pretreatment of carp fish scales. The dry weight of scales and solution ratio is 1/20 or 1/8. The pretreatment was carried out for 24 hours or 8 hours depending on the concentrations and type of alkalis. Acids including HCl, H_2SO_4, and H_3PO_4 with different concentrations have been chosen to demineralize for fish scale with the ratio of scale/solution of 1/20. After pretreatment and demineralization, the demineralized scales were immersed in 0.5 M acetic acid solution to extract the collagen. The authors combined the mechanic stirring with ultrasonication to optimize the collagen extract yield. The collagen solution was filtered and NaCl 10 % was used to precipitate collagen. To purify collagen, the raw collagen was dialyzed in distilled water for 48 hours using an osmosis membrane tube. In this study, the temperature of the experiment was kept at 4 ^{o}C to limit the denaturation of ASC.

Lingzhao and Blanco optimized the conditions for extraction of ASC from grass carp skin (*Ctenopharyngodon Idella*) or small-spotted catshark skin (*S. canicula*) by response surface methodology [35, 36].

Table 1. Summary of the kind of fish by-products, experimental conditions, and yield of collagen extraction by chemical method.

Source of collagen	Pretreatment	Extraction solvent	Extraction conditions	Yield	Reference
Redfish skins	NaCl 1.0 M	Acetic acid 0.5 M	Time = 24 hours Temperature = 4 ^{o}C S/L = 1/100 (w/v) stirring	47.5 %	[6]
Redfish scales	NaCl 1.0 M EDTA 0.5 M	Acetic acid 0.5 M	Time = 24 hours Temperature = 4 ^{o}C S/L = 1/100 (w/v) Stirring	6.8 %	[6]
Redfish bones	NaCl 1.0 M EDTA 0.5 M n-hexane	Acetic acid 0.5 M	Time = 24 hours Temperature = 4 ^{o}C S/L = 1/100 (w/v) Stirring	10.3 %	[6]
Skins of young and adult Nile perch	NaCl 0.8 M	Acetic acid 0.5 M	Time = 16 hours Temperature = 15 ^{o}C S/L = 1/20 (w/v) Stirring	63.1 % (young) 58.7 % (adult)	[7]
Carp fish scales	NaCl 1.0 M EDTA 0.5 M	Acetic acid 0.5 M	Time = 48 hours Temperature = 4 ^{o}C	9.79%	[8]
Carp fish scales	NaOH 0.1 M $Ca(OH)_2$ saturation HCl 0.2 M, H_2SO_4 0.5 M	Acetic acid 0.5 M	Time = 32,3 hours Temperature = 4 ^{o}C S/L = 1/20 (w/v) Stirring, ultrasonication	13.6 %	[10]
Skins, bones, fins	NaOH 0.1 N Butyl alcohol 10 % EDTA 0.5 M	Acetic acid 0.5 M	Time = 96 -120 hours Temperature = 4 ^{o}C Stirring	49.8 - 51.4 % (skin collagen) 40.1 - 53.6 % (bone collagen) 5.2 -36.4 % (fin collagen)	[31]
Carp fish scales	NaCl 10 wt.% HCl 0.4 M	Acetic acid 0.5 M	Time = 73.5 hours Stirring	-	[32]
Grass carp skin	NaOH 0.05 -0.1 M Butyl alcohol 10 % (v/v)	Acetic acid 0.5 M	Time = 108 hours Temperature = 4 - 20 ^{o}C Stirring	46 - 91 %	[33]
Grass carp skin	NaOH 0.1 M Diethyl ether	Acetic acid 0.54 M	Time = 62.1 hours Temperature = 4 ^{o}C Stirring	19.7 %	[35]

Small-spotted catshark skin	NaOH 0. 1 M	Acetic acid 1 M	Time = 36 hours Temperature = 4 - 8.3 ^{o}C Stirring	34.22 %	[36]

Three independent variables consist of acetic acid concentration, temperature, and time. The object function is collagen yield. The authors found the optimal conditions for extraction of the ASC were the acetic acid concentration of 0.54 M, at 24.7 ^{o}C and extraction time of 32.1 hours. The predicted yield of ASC at the optimal condition was 19.7 % [35]. Blanco *et al.* found the optimal conditions for the extraction of collagen from the small - spotted catshark skin (*S. canicula*) were temperature at 4 ^{o}C, for 2 hours, and a concentration of NaOH of 0.1M for the pre - treatment process at 25 ^{o}C, a time of 34 hours and a concentration of acetic acid of 1 M for the collagen extraction process [36]. Table 1 summarizes the kind of fish by-products, experimental conditions, and yield of collagen extraction by chemical method.

2.2. Biochemical method for fish collagen extraction

Table 2. Summary of the kind of fish by-products, experimental conditions, and yield of collagen extraction by biochemical method.

Source of collagen	Pretreatment	Extraction solvent	Extraction conditions	Yield	Reference
Carp fish scales	NaCl 10 wt.% HCl 0.4 M	Acetic acid 0.5 M Pepsin 0.5 % w/v	Time = 97.5 hours Stirring	-	[32]
Red drum fish	$NaHCO_3$ 30 % EDTA-HCl 0.5 M	Acetic acid 1 M Pepsin 0.5 % w/w	Time = 14 hours Temperature = 4 ^{o}C S/L = 1/10 (w/v) Stirring	4.32 %	[38]
Pangasius skins	Citric acid 0.25 %	Deionized water Alcalase 2 % w/v	Time = 22 hours Temperature = 55 - 65 ^{o}C Stirring	15.07 %	[39]
Small-spotted catshark, swordfish, yellowfin tuna skins	NaOH 0.1N Butyl alcohol 10 %	Acetic acid 0.5 M Pepsin 0.1 % w/v	Time = 48 - 96 hours Temperature = 4 ^{o}C S/L = 1/10-1/40 (w/v)	14.16 - 61.17	[40]

Biochemical method is an efficient method to obtain fish collagen with a high protein extraction yield. The general principle of this method is using enzyme in combination with acid to obtain pepsin-solubilized collagen (PSC). Fengxiang *et al.* extracted by using acetic acid combined with pepsin enzyme to extract PSC [32]. Firstly, the fresh carp fish scales were pretreated with 10 wt.% of NaCl solution for 24 hours before with 0.4 M HCl solution for 90 minutes. These processes remove the fats and proteins in the surface of fish scales and minerals in the structure of fish scales. The fish scales were then immersed in 0.5 M acetic acid solution and digested with 0.5 % (w/v) pepsin for 72 hours at 4 ^{o}C. Next, the extracts were centrifuged for 30 minutes to obtain supernatants which were used to salt out by introducing NaCl to form a 0.7 M final concentration. The collagen was then separated by centrifugating for 30 minutes. The solid was redissolved in 0.5 M acetic acid solution to precipitate with NaCl again. Finally, the raw collagen was dialyzed and freeze-dried to obtain PSC. In another publication, the

suitable pepsin enzyme was found at 2 wt.% compared to the weight of fish scales [37]. In this case, the weight of dry collagen reached 1.145 g per 150 g of fish scales. Sijin *et al.* extracted PSC from the red drum fish scale [38]. The pretreatment was carried out with 30 % $NaHCO_3$ solution and 0.5 M EDTA-HCl solution. The PSC was extracted by using 1.0 M acetic acid containing 0.5 % (w/w) of pepsin. The ultimate yield of PSC was 4.32 ± 0.30 % (dry wt.).

Recently, collagen peptides which are molecular weight lower than 20 kDa were produced from some types of fish skins by enzymatic hydrolysis to cut the collagen chains. Azizah *et al.* extracted collagen peptides from *Pangasius* fish skin using 2 % alcalase [39]. The gelatin yield reached 15.07 ± 1.45 % (w/w). The gelatin was then hydrolyzed by alcalase at 55 °C for 180 minutes with a degree of hydrolysis of 48.06 ± 1.97 %. Blanco *et al.* extracted PSC using pepsin from the teleost skins and chondrocytes skins yielded 14.16 % and 61.17 %, respectively [40].

2.3. Other methods for fish collagen extraction

Ultrasound-assisted extraction is one of efficient methods for the extraction of collagen. Sonication pretreatment can be used before collagen extraction to increase the extractability of the collagen [41 - 43]. Zou *et al.* indicated that the yield of collagen extraction from soft-shelled turtle calipash reached 16.3 % when using ultrasound pretreatment [41]. Petcharat *et al.* extracted collagen from clown featherback skin using an ultrasound-assisted process with a high yield extraction, from 23.46 - 35 % [43].

The extrusion-hydro-extraction process was applied to extract collagen from tilapia (*Oreochromis sp.*) fish scales (TFS) [44]. The advantages of this method are the shorter time for the extracting process and higher protein extraction yield in comparison with chemical methods. Firstly, fish scales were mixed with double-distilled water (ddwater), citric acid, and acetic acid at the ratio of 4.7/1 for the preconditioning step. Next, the mixture was extruded at 135 ^oC to obtain the TFS powder. This powder was then soaked in ddwater at a ratio of 1/10 (w/v) and shaken at 25 ^oC or 50 ^oC for 1 hour. The supernatant was obtained by centrifuging before being lyophilized. The highest extraction yield of water-soluble collagen reached 12.3 %.

Sustainable deep eutectic solvents (DESs) and acids have been used for separating collagen peptides from fish by-products [45, 46]. Bai *et al.* investigated six kinds of DESs for collagen extraction from cod skins. Using choline chloride – oxalic acid (ChCl-OA) DES is to reach high extraction efficiency and purity. The optimal technological parameters include the ChCl – OA molar ratio of 1/1, extraction temperature of 65 ^oC, contact time of 2 hours, and solvent-to-solid ratio of 80/1 ($mL.g^{-1}$) were selected to extract higher molecular weight collagen peptides while the optimal conditions for extraction of lower molecular weight collagen peptides were ChCl – OA (1/1)/cod skins (120/1 mL/g) at 65 ^oC for 6 hours. The yield of the collagen extraction process reached 91.57 - 96.01 % and the purity of collagen was up to 93.14 - 100 % at the optimal conditions [45].

3. CHARACTERISTICS OF FISH COLLAGEN

Collagen extracted from fish re-products, including skins, scales, bones, and fins, is the type I collagen with a fibril structure, consisting of two different α chains. Depending on extraction conditions and methods, the properties and characteristics of obtained collagen are different. Zhang *et al.* compared the properties of ASC and PSC extracted from waste freshwater carp fish scales. By sodium dodecyl-sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis, both ASC and PSC have two α chains with a molecular weight of 117.3 kDa and 107.4

kDa. They contained 18 amino acids with a high content of proline (Pro), glycine (Gly), and hydroxyproline (Hyp), characterized for $(Gly\text{-}Pro\text{-}Hyp)_n$ triple helix chains of collagen. The slight variation in the amino acid composition of ASC and PSC, for example, Gly content in PSC, ASC was 302, 306 residues/1000, Pro content in PSC, ASC was 122 and 110 residues/1000, Hyp content in PSC, ASC was 109 and 89, respectively, is caused by the different extraction method. The degrees of hydroxylation of proline for PSC and ASC were 47.2 % and 44.7 %, respectively. The PSC has a denaturation temperature lower than ASC may be due to the enzyme hydrolysis action, the denaturation temperature of PSC was 29.0 ^{o}C while that of ASC was 32.9 ^{o}C. Collagen absorbed ultraviolet (UV) at 233 nm. There were two diffraction peaks in the X-ray diffraction (XRD) of collagen, at about 7.44^{o} and 19.78^{o} that were assigned to the ordered structure or ordered structure snippet of proteins [32].

When using the carp (*Cyprinus carpio*) fish scales as a source for the extraction of collagen, the technology factors had a distinguished effect on the properties of obtained ASC types. Prashant *et al.* and Chinh *et al.* used different reagents for the pre-treatment of fish scales and the total time for extraction was also varied. Although ASC types exhibited the fibril structure as well as amide A, amide B, amide I, amide II, and amide III group vibrations of type I collagen, contained two α chains (Figure 5), their denaturation temperature was different. ASC extracted by Prashant *et al.* had a denaturation temperature of 37 ^{o}C [8] while Chinh *et al.* extracted ASC with a denaturation temperature of 32.2 ^{o}C [10].

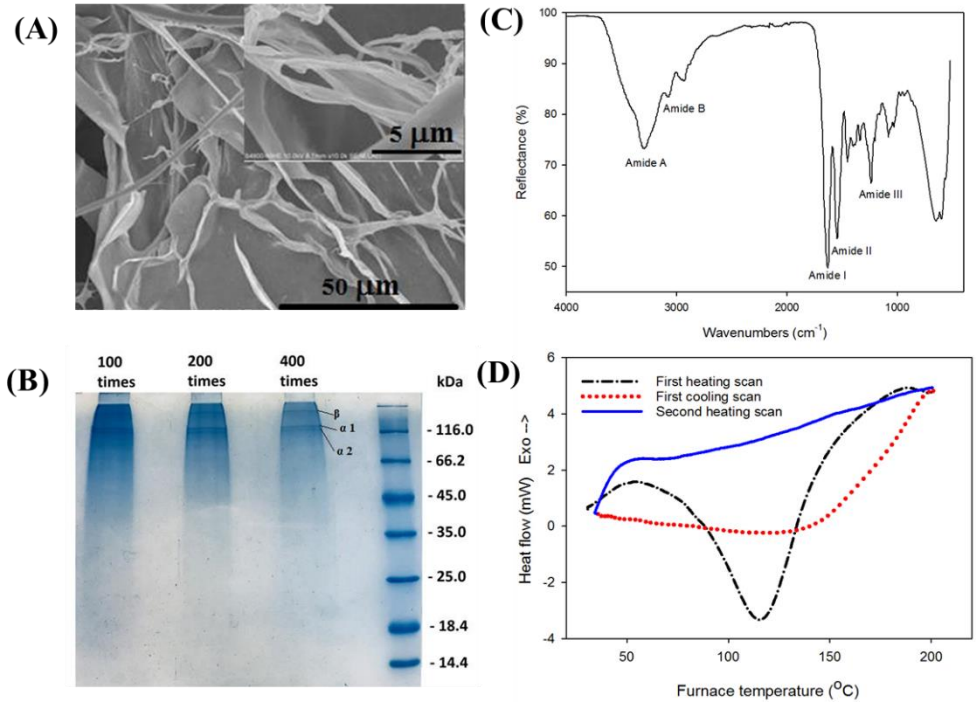

Figure 5. Some characteristics of collagen extracted from fish scales: (A) SEM image, (B) SDS-PAGE pattern, (C) IR spectrum, and (D) DSC diagram. Adapted from Ref. [10]. Copyright © 2019, SAGE Publishing.

The properties of collagen were also influenced by the type of fish re-products. Lin *et al.* found the difference among collagens obtained from the skins, scales, and bones of deep-sea redfish (*Sebastes mentella*) [6]. According to that, the bone collagen had a denaturation

temperature lower than scale collagen but higher than skin collagen. These collagens contain eighteen amino acids, with the content ordered glycine, alanine, proline, glutamic acid, hydroxyproline, serine, aspartic acid, arginine, lysine, valine, threonine, leucine, phenylalanine, methionine, isoleucine, hydroxylysine, tyrosine, and histidine. The variation in amino acid content of skin, scale, and bone collagen is small. Glycine was the most abundant amino acid in these collagens. The estimated molecular weight for α chains of these collagens was quite high, from 120 - 150 kDa. In another literature, among collagens extracted from fish skins, bones and fins, the bone collagen had the highest denaturation temperature, from 29.5 - 30.0 ^{o}C, followed was the fin collagen, from 28.0 - 29.1 ^{o}C and skin collagen, from 25.0 - 26.5 ^{o}C [31]. Duan also confirmed that the collagens from the skin, bone, and scale of carp (*Cyprinus carpio*) are type I collagen with two α1 and one α2 chains [47]. The molecular weight of α2 chain was 116 kDa. These collagens have denaturation temperatures around 28 ^{o}C. Collagens extracted from Fish sardine *Sardinops melanostictus*, Japanese sea bass *Lateolabrax japonicus*, and red sea bream *Pagrus major* also have three α chains in the fibril structure and the denaturation temperature of 28.0 - 28.5 ^{o}C [48].

The living environment and body temperature have a strong effect on the properties of obtained fish collagens, especially the denaturation temperature. The denaturation temperature of collagen extracted from temperate and tropical fish species is often higher than that from cold - water fish. A hypothesis for this difference is the low content of amino acids, extending a lower degree of hydroxylation or hydroxyproline, an important factor in maintaining the stability of trimmers incollagen [6, 10].

Sankar *et al.* made collagen sheets from fish scales and characterized their properties [49]. Besides the presence of vibrations of amide groups (amide A, B, I, III) in collagen, the peak characterized for vibration of amide II was absent while peaks assigned to phosphate stretching, P-H bending that is due to the calcium salts were observed [49]. The collagen sheets were degraded by heat and followed three steps, loss of water and residues, loss of amino acids and break of α chains, loss of β chains and inorganic substance or the combustion of carbon [10, 49]. The collagen sheets had a low tensile strength, 2 MPa, which was enough to use as a wound dressing material [49].

Collagen extracted from scales, skins, and bones of bigeye tuna (*Thunnus obesus*) by chemical method had a great solubility in acetic acid 0.5 M solution at pH 6 while the pepsin collagens displayed the maximum solubility at pH 5. The solubility of collagen reduced suddenly in the case of pH 7 due to the increase in the hydrophobic-hydrophobic interaction among molecules of collagen, and then increased slightly at pH 8 - 10 because of the repulsive effect of the collagen molecules. The point of zero charges of these collagen solutions ranged from 5.4 - 6.4 [50]. These values are similar to the point of zero charges of collagens extracted from brown-banded bamboo shark skin (6.2-6.6) [51], clown featherback skin (5.5 - 5.7) [52] but much lower than those of the snakehead scale [53].

4. APPLICATIONS OF FISH COLLAGEN IN DRUG DELIVERY SYSTEMS

Marine collagen sources in general and fish collagen in particular have very potential for applications in drug administration and delivery, wound healing, skin and regeneration, tissue engineering, the food industry and cosmetics, etc. (Figures 6 and 7). Among them, the research related to drug delivery and wound healing accounts for the majority.

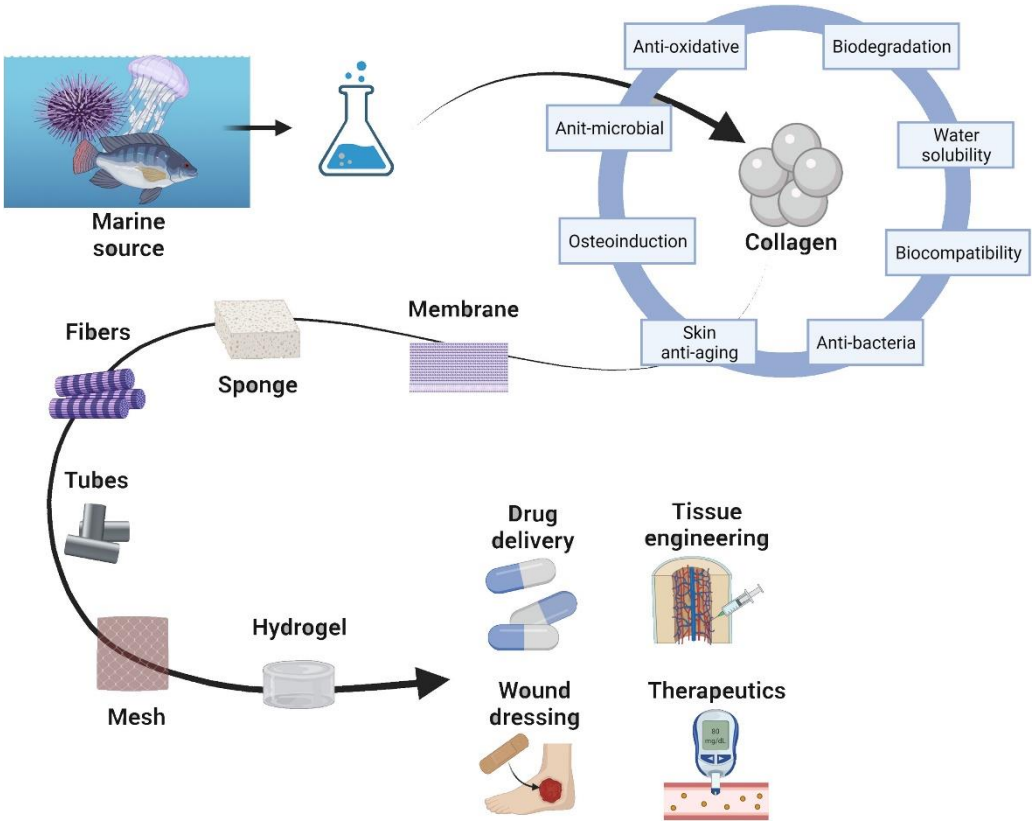

Figure 6. Applications of marine collagen sources. Adapted from Ref. [21]. Copyright © 2022, Elsevier B.V. Publishing.

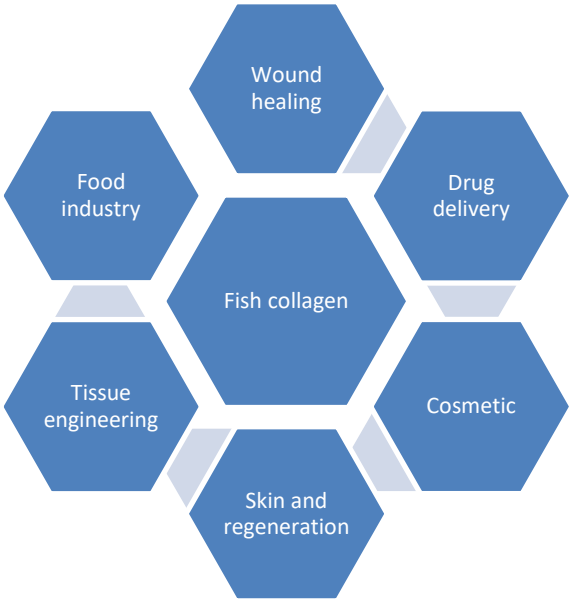

Figure 7. Some applications of fish collagen.

In the drug delivery field, fish scale collagen was combined with carrageenan for loading allopurinol. The solution, 3D printing, and gelation methods have been applied for the preparation of the fish scale collagen-based biomaterials. The size of allopurinol dispersed in carrageenan/fish scale collagen film ranged from 50 - 100 nm while the carrageenan/fish scale collagen/allopurinol particles are spherical and have a size from 150 - 800 nm. The suitable weight ratio of carrageenan and fish scale collagen for preparation of carrageenan/fish scale collagen/allopurinol particles is 1/1. The drug loading efficiency of carrageenan/fish scale collagen depends on the component ratio, preparation condition, and method. The solubility of allopurinol in simulated intestinal fluid (SIF) and simulated gastric fluid (SGF) was improved significantly in case of loading on carrageenan/fish scale collagen, increased 3.27 - 7.25 times as compared to crystal allopurinol. The release of allopurinol from carrageenan/fish scale collagen/allopurinol biomaterials was followed two steps: fast release and stable, controlled release. After 32 hours of testing, the allopurinol content from the biomaterials reached 19.65 - 67.65 % in SGF and 23.33 - 98.08 % in SIF. The release of allopurinol from the biomaterials complied with Korsmeyer – Peppas model, through a complex process, including swelling and dissolution of polymers, diffusion of drug, interactions between drug-drug, drug-polymers, polymer-polymer, etc. [54 - 57].

Fish scale collagen was also combined with chitosan for loading lovastatin. By ionic gelation method, the biomaterials based on chitosan, fish scale collagen, and lovastatin were prepared in a spherical shape with a size ranged from 72.1 to 444.0 nm. The biomaterials had a high drug loading efficiency, from 89.0 to 99.2 %, depending on the content of lovastatin. The fish scale collagen content, pH, and lovastatin content influenced the lovastatin content released from the biomaterials. The drug interacted with polymers through the hydrogen bonding between hydroxyl and carbonyl groups of lovastatin and hydroxyl, amide groups of chitosan, and collagen. These biomaterials did not cause acute toxicity to mice at the used dose of 5000 $mg.kg^{-1}$ and did not influence on functions of mice after 28 days of sub - chronic toxicity testing. The fish scale collagen based on biomaterials are potential for drug delivery application [58].

Fish scale collagen is used as a main composition in the bio-complexes by a combination of it with bioactive compounds such as polyphenols (extracted from *Camellia chrysantha* leaf) and ginsenoside Rb1 (extracted from panax notoginseng root powder) [59, 60]. The bio-complex based on fish scale collagen, konjac glucomannan, *Camellia chrysantha* polyphenols and ginsenoside Rb1 had an average particle size of 115.4 - 593.0 nm with excellent inhibition of cancer cells (epithelial carcinoma - KB, lung cancer - LU1, liver cancer - Hep G2, breast cancer - MCF7, skin cancer - SK-Mel-2 cells), anti-inflammatory and antioxidant bioactivities. The inhibition 50 percent of cell growth, IC_{50} values of the bio-complex with KB, LU1, Hep G2, MCF7, SK-Mel-2 cancer cells were 18.01, 42, 64, 64, 47 $\mu g\ mL^{-1}$, respectively. The bio-complex did not cause toxicity on HEK-293 normal cells [59].

Fan *et al.* prepared the hydrogels based on tilapia skin collagen and chitosan and studied the factors that affected this preparation, including pH of the environment and the concentration of two components. At the neutral pH, the residual mass rate of the hydrogels was 29.1 % in 36 hours while at pH 6 and 5, the residual mass rate of the hydrogels was 8.4 % and 0 %, respectively. The suitable content of tilapia skin collagen and chitosan was 10 $mg\ mL^{-1}$ to form a stable hydrogel. Two model nanobodies, including 2D5 and KPU gene, were chosen for loading on the hydrogel. When increasing the concentration of nanobodies in the hydrogels, the cumulative release rate of 2D5 decreased while that of KPU increased. At pH 5.5, the release rate of 2D5 from the hydrogel reached the maximum value, 68.3 %, followed by pH 6.8 and 7.4. For KPU, the same trend was observed for the release rate at pH 5.5, 6.8, and 7.4 and the release

rate was ordered to pH 5.5 > pH 6.8 > pH 7.4. The hydrogels based on tilapia skin collagen and chitosan are promising for use in cancer therapy [61]. The scaffold materials based on fish scale collagen, chitosan, hydroxyapatite, and beta-tricalcium phosphate were prepared by freeze drying method and incorporated with ginsenoside compound K. The obtained materials have a highly biocompatible, highly porous, low swelling, and highly stable, potential for bone tissue engineering application [62]. In another report, the scaffold-controlled release systems for skin tissue engineering based on fish collagen, chitosan, and chondroitin sulfate were fabricated by freeze drying method and incorporated with fibroblast growth factor - loaded poly(lactide-co-glycolide) microspheres. After 28 days of testing, the cumulative release rate of proteins from the scaffold materials reached 40 - 70 % depending on the ratio of collagen and chitosan components as well as the size of microspheres [63]. In the report of Seo *et al.*, fish scale collagen encapsulated negative surface modified nanoliposome was fabricated to enhance the topical delivery of fish scale collagen as anti-aging cosmetics. The liposomes had a negative charge surface of - 44 mV, an average size of 175 nm, and an encapsulation efficiency of 11 % [64]. Both ASC and PSC extracted from the outer skin waste of marine eel fish (*Evenchelys macrura*) are used to prepare gel and films by solvent casting method for loading antibiotic drug delivery. Some model antibiotics, for example, ampicillin and tetracycline, were used and hydroxypropyl methyl cellulose E15 was used as an adhesive agent. The fish collagen could carry antibiotics against human pathogenic (bacteria and fungi) microorganisms [65]. Fish scale derived collagen combined with nanocellulose has also been used for loading lidocaine for controlled skin permeation. The lidocaine permeation rate increased from 2.5 to 7.5 % w/w after 36 hours of testing and reached a pseudo steady state profile from 5.0 to 10.0 % w/w [66].

5. APPLICATIONS OF FISH COLLAGEN IN WOUND HEALING

Skin is the largest organ in the body, consisting of three layers, the epidermis, dermis, and hypodermis. Collagen is the main structural protein in the skin. Therefore, one of the promising applications of fish collagen is for wound healing, especially for burn wounds or skin regeneration. Fish scale collagen has been used in the preparation of nanogels incombination with hydroxypropyl methyl cellulose and curcumin for wound healing applications. The nanogels were made by adding slowly fish scale collagen to hydroxypropyl methyl cellulose formulations with a suitable oil/surfactant mixture/water component ratio of 7.63/68.70/23.66 % w/w. The nanoemulsion of curcumin had a size of a droplet of 123.5 nm, a refractive index of 1.33 ± 0.01 RI, a viscosity of 99.07 ± 1.0 cP and a drug content of 99.32 ± 1.52 %. The nanogels loading nanoemulsion with the ingredients including curcumin 1 % w/w, fish scale collagen 1 % w/w, hydroxypropyl methyl cellulose 1 % w/w, oleic acid 7.68 % w/w, Tween80 34.35 % w/w, ethanol 34.35 % w/w, triethanolamine 0.05 % w/w, distilled water 23.66 % w/w. The above nanogel had a pH of 6.98 ± 0.20, spreadability of 7.2 ± 1.5, drug content of 99.20 ± 1.50 %, viscosity of 233.1 ± 50.12 cP, flux of 12.83 ± 4.20 μg cm^{-2} h^{-1}. The nanogels had high stability, high wound contraction, and were safe for dermatological application [67]. Shi *et al.* used both porcine skin-derived collagen and fish scale-derived collagen (FSC) for the fabrication of highly porous sponge scaffolds. The scaffold based on FSC had higher water uptake ratio, and water vapour transmission rate as compared to that based on porcine skin-derived collagen. The high-water vapour transmission rate is to produce a moist healing environment for wounds. These scaffolds were non-toxic to L929 fibroblast cells. When used for the treatment of burn wounds on rabbits, these scaffolds did not place any scars around the wounds [68]. The nanofibers based on fish collagen and bioactive glass have been prepared by electrospinning technique. The nanofiber mats had a high tensile strength, 21.87 ± 0.21 MPa and can inhibit the growth of *S. aureus*.

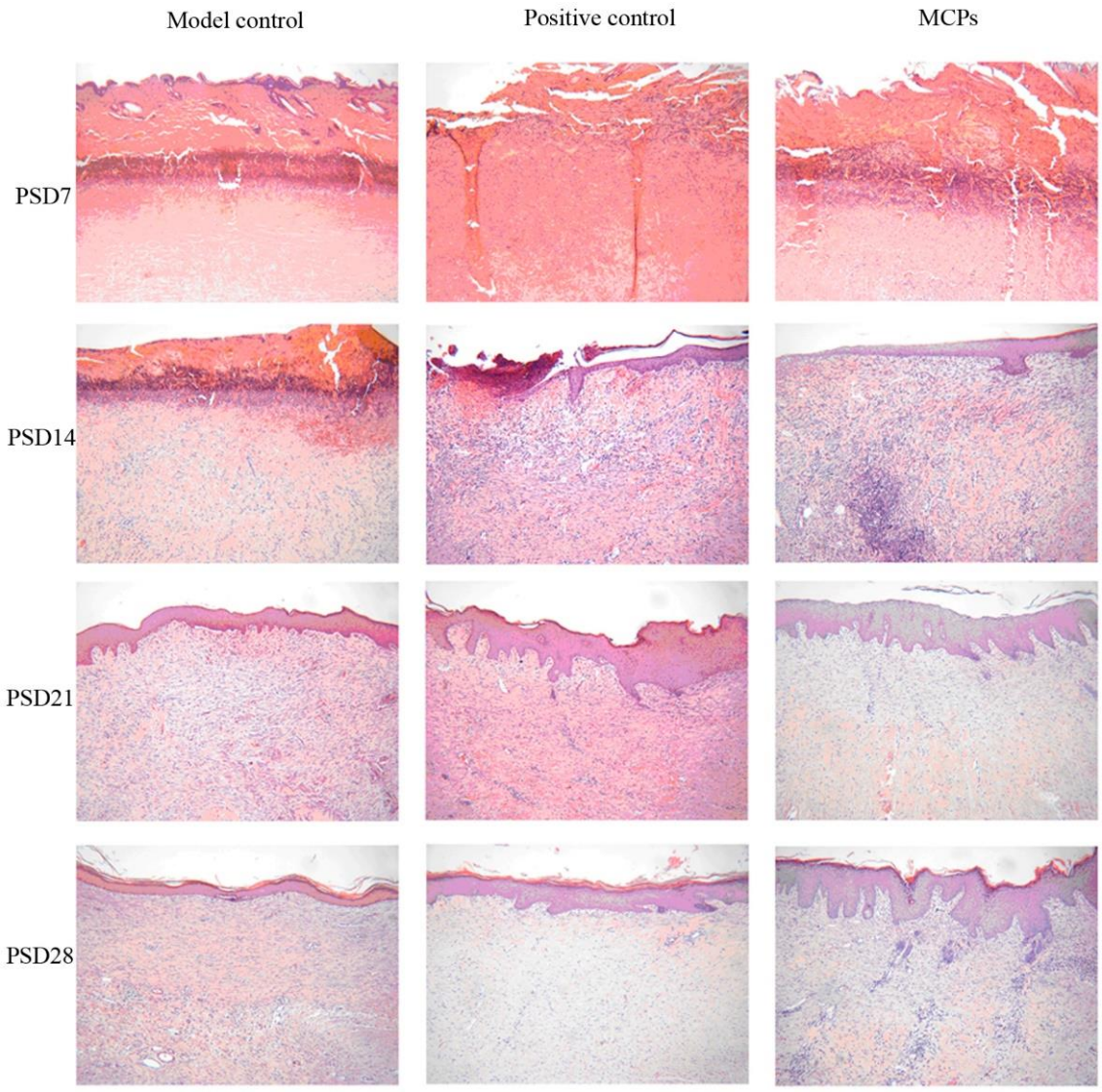

Figure 8. Micrographs of wound tissues in rabbits changed vs time (7, 14, 21, and 28 days) (H&E, 100x). Model control was a rabbit group without treating after scalding, positive control was a rabbit group treated with moist scald ointment, and MCPs was a rabbit group treated with marine collagen peptides extracted from fish skin. Adapted from Ref. [72]. Copyright © 2017, MPDI Publishing.

These mats can promote the adhesion, proliferation, and migration of human keratinocytes, they can accelerate rat skin wound healing [69]. In another report, biomimetic tilapia collagen sponge and nanofibers were developed for wound dressing. The collagen nanofiber mat had a contact angle of 21.2^{o}, tensile strength of 6.72 ± 0.44 MPa, composed of multiple amino acids, and had a weight loss temperature of 300 ^{o}C. Thanks to its great properties, these mats could promote the viability of human keratinocytes and human dermal fibroblasts, leading to skin regeneration rapidly and effectively [70]. Collagen extracted from the marine fish skin was combined with gelatin, poly(3-hydroxybutyric acid) and bioactive *Coccinia grandis* extract to fabricate a nanofibrous scaffold for wound dressing application. When treated with this scaffold, the hexosamine, hydroxyproline, and uronic acid levels increased, leading to the deposition of

collagen synthesis and reepithelialization accelerated. This is due to the presence of fish collagen and *Coccinia grandis* extract in the scaffold helped to accelerate the wound healing and to reduce the inflammation in wound healing. The fish collagen coated nanofibrous scaffold with bioactive compounds enhanced the faster healing of wounds [71]. Hu *et al.* also confirmed the potential of marine collagen peptides (MCPs) from the skin of Nile tilapia (*Oreochromis niloticus*) in the enhancement of the process of wound healing [72]. Thanks to the *in-vivo* on the rabbits, MCPs exhibited a high wound healing rate with the wound healed 100 % after 24 days of treatment. The histological findings indicated that when treated with MCPs, the wound on the tested rabbits was covered completely by new epidermis while inflammatory region was absence and mature granulation tissue proliferation appeared in dermis layer (Figure 8). The collagens from fish skin could also accelerate wound healing [73]. In addition, fish collagen can combine with bioactive traditional medicines to form a synergetic effect on the acceleration of wound healing and reduction of inflammation in wound healing [28, 74 - 76]. All the research highlighted the promise of fish collagen in wound healing applications.

6. RESEARCH DIRECTIONS IN COMING TIME

Fish collagen has many unique features and is gradually being used as a substitute for land-based animal collagen. Optimization for the extraction process of fish collagen from by-products (skins, scales) of some fish species (grass carp, catshark) has been carried out using response surface methodology. However, there are many species of fish as well as other fish by-products (fins, bones, etc.). Moreover, the living environment also influences on the composition of amino acids in fish collagen. Therefore, the optimization should be performed for fish collagen extraction with a variety of variables. The research to scale up the production of fish collagen from some sources of by-products containing less collagen such as scales, and bones is necessary. In particular, the *in-vivo*, clinical tests and trials for new fish collagen also need to be carried out to apply these new products in fields requiring high quality and safety. The combination of fish collagen with other biopolymers and bioactive compounds for applications in drug administration and delivery, wound healing, etc. is a useful strategy attracting to study and development.

7. CONCLUSION

Fish collagen could be extracted from fish by-products consisting of skins, scales, fins, and bones by chemical or biochemical methods or others. The extraction of fish collagen was influenced by extraction method, a ratio of solid phase/liquid phase, the concentration of substances, nature of substances, extraction time, extraction device, extraction temperature, etc. Fish collagen is collagen type I with a fibril structure, has good adsorption ability, easy absorbance, good biocompatibility, and biodegradability. Therefore, fish collagen is definitely a promising biomaterial for applications in many fields of living and industry, especially, for drug administration and delivery, hemostatic, and wound healing. However, the optimization of the extraction process as well as giving scale up to industry are necessary to form fish collagen types having a suitable cost, good quality, and great performance. From that, the applications of fish collagen would be spread in a variety of fields.

CRediT authorship contribution statement. Nguyen Thuy Chinh: Methodology, Writing manuscript, Conceptualization, Formal analysis. Thai Hoang: Supervision, Conceptualization, Review and editing, Formal analysis.

Declaration of competing interest. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

ABBREVIATION

ASC: Acid-soluble collagen
CL: Collagen
CS: Chitosan
DCM: Dichloromethane
DSC: Differential scanning calorimetry
EDTA: Ethylenediaminetetraacetic acid
FESEM: Field emission scanning electron microscopy
FSC: Fish scale-derived collagen
GI: Gastrointestinal
HPLC: High Performance Liquid Chromatography
HPMC: Hydroxypropyl methylcellulose
IR: Infrared
PCL: Polycaprolactone
PEG: Polyethylene glycol
PEO: Polyethylene oxide
PSC: Pepsin-solubilized collagen
SDS-PAGE: Sodium dodecyl-sulfate-polyacrylamide gel electrophoresis
SEM: Scanning electron microscopy
SBF: Simulated body fluid
SGF: Simulated gastric fluid
SIF: Simulated intestinal fluid
TEM: Transmission electron microscopy
TGA: Thermo-gravimetric analysis
UV-Vis: Ultra Violet-Visible Spectroscopy

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