IN-VESTIGATING FISH AS AN INDEGINOUS SOURCE FOR DEVELOPING GELATIN BIO-MATERIAL IN TISSUE ENGINEERING APPLICATION – A REVIEW

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***Abstract* — Until recently, bovine and porcine sources were the most commonly utilized and preferred sources for the production of gelatin biomaterial for tissue engineering applications. Manufacturers have begun to explore for alternate sources that bridge this limitation while still meeting the qualities necessary. With a greater emphasis on overcoming the limitations presented by these sources and because of the ever-increasing demand for biomaterials to meet the growing demand in tissue engineering applications, this article investigates the various extraction processes of gelatin biomaterial from an indigenous source such as fish in order to expose the possibility of technology extraction technology and modification in its properties to suit tissue engineering applications.**

Keywords — *biomaterials, collagen, gelatin, fish, polymers*

I. INTRODUCTION

Tissue Engineering

"The developing field of tissue engineering (TE) aims to regenerate damaged tissues by combining cells from the body with highly porous scaffold biomaterials, which act as templates for tissue regeneration, to guide the growth of new tissue" [1].

The term "tissue engineering" was formally conceived at a National Science Foundation workshop in 1988 as "the application of principles and methods of engineering and life sciences toward the fundamental understanding of structure-function relationships in normal and pathological mammalian tissues and the development of biological substitutes to restore, maintain or improve tissue function" [2].

The main challenge for tissue engineering is represented by the choice of appropriate materials for scaffold production. It is important when planning or determining the sustainability of a scaffold to assess that it satisfies the following key requirements: (i) biocompatibility, (ii) bioactivity, and (iii) biodegradability [2].

In tissue engineering, biomaterial is used because it ideally degrade at a comparable rate to growth of new tissue at the site of implantation. Collagens constitute the primary structural element of the ECM and provide tensile strength, regulate cell adhesion, support chemotaxis, cell migration and direct tissue development [3]. Cells thus actively interact with the ECM via various surface receptors and these interactions can be of high importance to maintain cell functions. Unsurprisingly, gelatin, as a collagen derived product which can be considered as a biomaterial, can play a major role in tissue engineering at various steps of the process [3].

Biomaterials

A biomaterial is used to make devices to replace a part or a function of the body in a safe, reliable, economic, and physiologically acceptable manner [4]. A variety of devices and materials presently used in the treatment of disease or injury include such commonplace items as sutures, needles, catheters, plates, tooth fillings, etc. [4]. Over the years, various definitions of the term biomaterials have been proposed. For example, a *biomaterial* can be simply defined as a synthetic material used to replace part of a living system or to function in intimate contact with living tissue. The Clemson University Advisory Board for Biomaterials has formally defined a biomaterial to be "a systemically and pharmacologically inert substance designed for implantation within or incorporation with living systems [5]." Black defined biomaterials as "a nonviable material used in a medical device, intended to interact with biological systems" [6]. Other definitions have included "materials of synthetic as well as of natural origin in contact with tissue, blood, and biological fluids, and intended for use for prosthetic, diagnostic, therapeutic, and storage applications without adversely affecting the living organism and its components" and "any substance (other than drugs) or combination of substances, synthetic or natural in origin, which can be used for any period of time, as a whole or as a part of a system which treats, augments, or replaces any tissue, organ, or function of the body" [7], [6]. By contrast, a *biological material* is a material such as skin or artery, produced by a biological system. Artificial materials that simply are in contact with the skin, such as hearing aids and wearable artificial limbs, are not included in our definition of biomaterials since the skin acts as a barrier with the external world.

Synthetic polymers represent the largest class of **biomaterials** useful in applications in both, soft and hard tissue. They can be hydrophobic like silicone rubber (SR), polypropylene (PP), polyethylene (PE) and polymethyl-metacrylate (PMMA), or water swelling or even water soluble like polyethylene glycol (PEG). Some of them are degradable, others remain almost unchanged within the body. Polymers are long chained molecules consisting of a large number of small repeating units. They can be amorphous or semicrystalline and their surfaces may be modified chemically and biochemically. *PMMA*, to cite the most important polymer in current orthopedics, is a permanent bone substitute material which is frequently used to improve the anchorage of fracture fixation devices and joint replacement

prostheses. It is also used in vertebroplasty in severe cases of impact fractures of the vertebral body due to osteoporosis or neoplasm. Although this material has proven its usefulness in these applications, on the other hand it poorly osseointegrates even possibly disturbing bone healing and remodeling through its genuine inert properties. Additionally tissue necrosis may be caused through heat production up to 80°C while curing and by creating monomer toxicity. In combination with primary (micro-) mechanical instability these properties may lead to the formation of an interface membrane and subsequent aseptic loosening. Despite all those concerns, PMMA is still the most frequently used polymer bone cement in Europe. Hydrogels are novel polymers that gained more popularity in recent years. As an example for degradable water containing substances they can be injectable and of different water contents. They can consist of Poly-(ethylene glycol), or gelatin. They are used experimentally and clinically as biomaterials for the controlled release of bone regeneration activity enhancing substances like Transforming Growth factor (TGF)-beta 1, Insulin-like growth factor (IGF)-1 and bone morphogenetic protein-2. Furthermore, hydrogels can also be used as scaffolds and carriers for osteoprogenitor and other cells like chondrocytes, fibroblasts and mesenchymal stromal cells.

Gelatin as a Biomaterial

Gelatin is a naturally occurring macromolecular and biodegradable protein that is produced by the controlled partial hydrolysis of collagen synthesized from skins, white connective tissues and bones of animals which is composed of amino acid residues at different proportions and combinations [8]. As a source of protein-matrix of gelatin, collagen is the most naturally found protein in humans and animals. Collagen has been contained anywhere else in the body, but the skin, bones, tendons, and ligaments are the most abundant. Often in the process of consumption, the extraction of gelatin from collagen requires a boiling state or a hydrolysis reaction (sometimes enzymatic assisted) in order to produce a flavourless and colourless substance, famously known as a gelling agent in food production. According to [9] gelatin has a strong emulsifying power and may constrain the aggregation of milk, soybean milk and other proteins into gastric acid after entering the stomach, which is beneficial to food digestion. In general, two different types of gelatin may be produced based on the pre-treatment of collagen: type A gelatin and type B gelatin. Type A is an acid treatment gelatin, that is an isoelectric point at pH 6 to 9 and is most commonly used for the less covalently crosslinked collagen found in pig skin. Type B is an alkaline treatment gelatin which is an isoelectric point at pH 5 and can be applied to more complex collagen found in bovine hides.

Gelatin has increasingly been used in biomedicine beyond its traditional use in food and cosmetics. The appealing advantages of gelatin, such as its cell-adhesive structure, low cost, off- the-shelf availability, high biocompatibility, biodegradability and low immunogenicity, among others, have made it a desirable candidate for the

development of biomaterials for tissue engineering [10]. There are two main types of gelatin. Type A, with isoionic point. of 7 to 9, is derived using exclusively acid pretreatment. Type B, with isoionic point of 4 to 5, is the result of an alkaline pretreatment [11].

In the bio-medical industries, gelatin is used to produce substrate known as extracellular matrix (ECM) which helps to facilitate the grown of cells and offers structural stability during tissue engineering process.

Gelatin Structure

Based on chemical composition, gelatin is different from collagen. In collagen, the triple helix structure consists of three α chains while the gelatin structure consists of three different chains. These chains are α chains, β chains, and γ chains. Gelatin is structurally composed of chains of 3 recurrent amino acids composed of Glycine-Proline-Hydroxyproline. The functional properties of gelatin and the stability of the triple helix is largely determined by the involvement of amino acids proline and hydroxyproline. [12]. Figure 1 shows the general structure of gelatin produced from various sources.

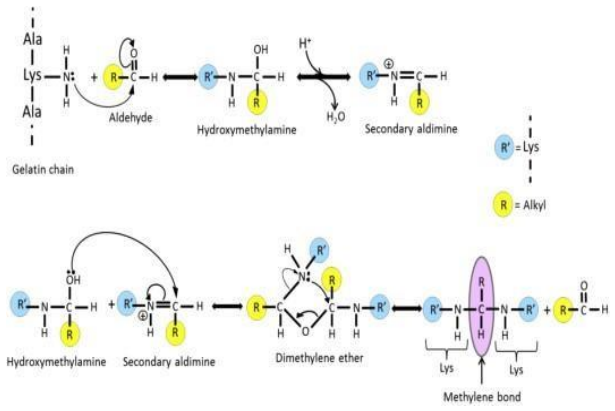

Figure 2. .Gelatin structure and composition (Anne *et al., 2014)*

Why Fish Gelatin

Among mammals, bovine and swine are the most common extraction sources. The reason lies in the fact that these two species are the highest consumed mammalian meats per capita in the United States.

However, the limitation due to socio-cultural and health-related concerns in other type of gelatin extracted collagen from pork and cow bones has revived the interest to discover the alternatives sources of gelatin from fish (marine and freshwater) and poultry as raw materials [13]. Both Judaism and Islam forbid the consumption of any pork-derived products, while Hindus do not consume cow-derived products [14]. Although bovine and porcine collagens cover most of the market size and tendon recover is easy, their use is limited because of immune response, zoonosis problems and religious constraints. Bovine and porcine collagen is particularly poor immunogen and their triple helical domains are highly homologous to human collagen, immunologically relevant differences lay in the telopeptide regions. Bovine collagen triggers immune reactions in about the 2%–4% of the World population. However, this sensitivity has been considered generally acceptable for tissue engineered implants for human use. Furthermore, the fact that up to 3% of the population manifests an inherent immunity, is enough to routinely perform allergy testing prior to material implantation. To this, two consecutive negative skin tests at 6 and 2 weeks are required

before any treatment. Among issues, the zoonosis transferring risk (e.g., the foot and mouth disease (FMD) and the group of the bovine spongiform encephalopathies (BSE), among which the most dangerous for humans is the transmissible spongiform encephalopathy (TSE)) is the most serious. Porcine collagen causes less allergic response but, just like the bovine source, the setback of zoonosis limited its use. Avian influenza is also a concern in a poultry-related product. Therefore, the extraction of gelatin from alternative sources, especially by-products from fish scales and bones should be taken into consideration.

The waste generated from the worldwide production and processing of shell-fish and fish scales is a serious problem of growing magnitude. This abundant waste may pose environmental [15]. The use of this waste for renewable products such as biopolymers is a dual-purpose opportunity. Therefore, the abundance sources of fish byproduct such as bones, scales and skin can be the great sources of gelatin. Fish scales and bones are more preferable in the extraction of gelatin because it yields large amount of gelatin due to high content of amino acids (proline) compared to fish skin [16]. Thus the aim of this review is to collect the various researches and work done on fish gelatin extraction as a biomaterial in tissue engineering application. **G**enerally, fish gelatin has benefits over artificial polymers in biomedical uses because of its accessible bioactive motifs in the polymer, high biocompatibility, and little immune response [17]. Additionally, gelatin comprises plentiful arginine–glycine–aspartic acid sequences, which encourage cell adhesion, lead to simplify extracellular matrix remodeling. [18]. It has low antigenicity, low gelling point, superb solubility but have a low mechanical modulus and undergoes rapid degradation [19]. To overcome these limitations fish gelatin was modified by cross-linking and/or the use of polymers [20]. Crosslinking can be physical, chemical or enzymatic [21]. Several cross-linking agents which can be used includes used (glutaraldehyde, phenolic acid, 1-Ethyl-3-(3-Dimethylaminopropyl) Carbodiimide (EDC), etc.). [22, 23]

The most important properties of gelatin are:

1. Thermoreversible gel formation
2. High mechanical strength.
3. Texturing
4. Thickening
5. High water binding capacity
6. Emulsion formation and stabilization
7. Foam formation
8. Protective colloidal function
9. Adhesion/cohesion.

II. Gelatin Extraction

During gelatin extraction, the gelatins prepared by the acid pretreatment are called type A gelatin, whereas those produced by the alkali pretreatment are called type B gelatin. The alkali process refers to a pretreatment with an alkali solution, in most cases followed by neutralization with an acid solution. Generally, alkali pretreatment is used mainly for hides and bones, which are stable and highly cross-linked materials. The acid pretreatment is applied to the less mature materials

containing a low concentration of intra and intermolecular crosslinks such as pig skin, bone of young cattle, fish skin and fish [24]. These different steps are summarized in Figure 1 below.

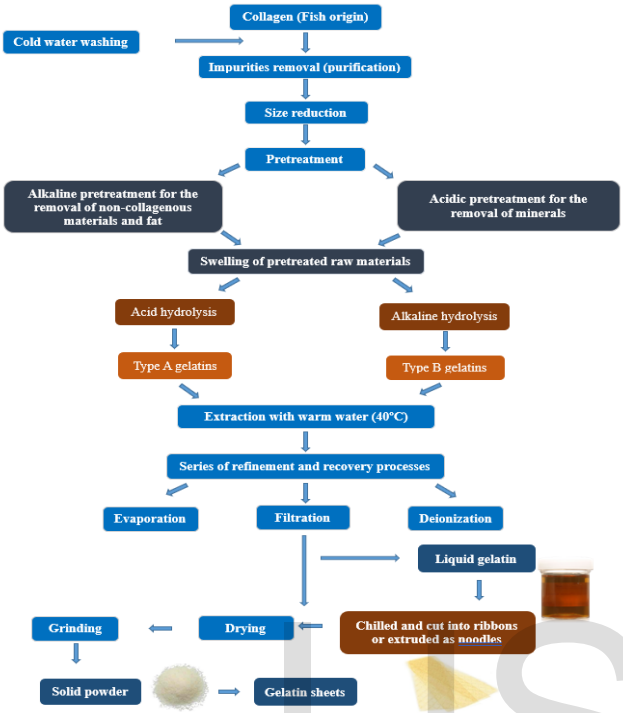

Figure 1. Preparation method of gelatin from collagen. **[25]**

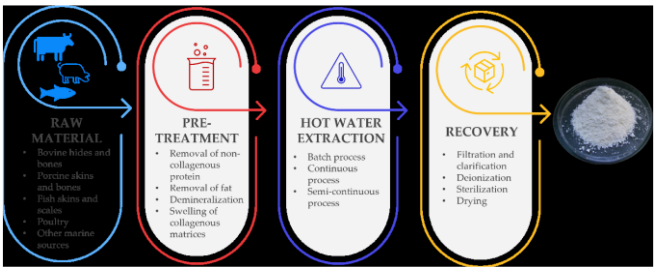

Figure 2. The general gelatin extraction procedure [26]

The review below enumerates the extraction routes adapted and consequently their properties for tissue engineering applications

Isriany *et al.* (2016) investigated the characteristics of Gelatin extracted from Milkfish (*Chanoschanos*) Scales and Bones with Variation in Acid and Base Concentrations. Scales and bond milkfish were pre-treated with 0.01 M, 0.1 M and 1 M of sodium hydroxide and acetic acid; extraction used water bath (60 °C, 8 hours), sonicator (50 °C, 3 hours), microwave (100 °C, 1 hour), and autoclave (121 °C, 1 hour); and drying used dry hot air (60 °C) and freeze dried (-40

° C). The characteristics of gelatin determined with measuring viscosity, pH and yield. The most optimal method ($p < 0.05$) for extraction of milkfish scales and bones used 1N acetic acid for 8 hours (pretreatment process), water bath 60°C, 8 hours for hydrolysis methods and freeze dried for the drying methods. The characteristics of gelatin like viscosity, pH and yield were 3.3890 cP, 4.6, and 1.9% respectively, while milkfish scales were 4.6759 cP, 5.4 and 3.7%. Gelatin produced from bone can be used for food and pharmaceutical ingredients (hard, soft capsule, and tablets) while those from scales can be used for food and pharmaceutical ingredients (hard capsule).

Merina *et al.* (2017) conducted research to study the physico-chemical properties of Gelatin from fish waste as an alternative source due to its increasing demand and its excellent biocompatibility, biodegradability properties in biomedical application**.** The gelatin was extracted from the scales of freshwater fish, *Labeo rohita*. After extraction, the proximate analysis and physicochemical analysis of the fish scale gelatin were carried out. This functional polymer was also characterized using different analytical methods, such as UV-vis spectroscopy, scanning electron

microscopy (SEM), and X-ray diffraction (XRD) for the evaluation of crystalline and surface morphology, and Fourier transform infrared spectroscopy (FTIR) for structural determination. The scales of *L. rohita* yield 24% (dry weight basis) of gelatin, indicating this fish species as potential source of gelatin. The proximate analysis determined was

low moisture content (4.2%), ash (1.4%) and high protein (90%) content. Table 2.1 shows the proximate and physico-chemical analysis of the extracted gelatin. The result of the study confirms the effectiveness of extraction method used. The fish scales of *L. rohita* are found to be a sustainable and renewable source of gelatin with desirable functionalities and it is the best alternative for mammalian gelatin in food and bio-medicine.

Table 2.1 The proximate and physico-chemical analysis of the extracted gelatin Merina *et al*., 2017).

Factors	**Fish scale gelatin**
Moisture	4.2%
Protein	90%
Ash	1.4%
Lipid	1.05%
pH	4.35
Melting temperature	27 °C

Anchana, *et al*., (2019) probed the physico-chemical properties of Gelatin extracted from *Carcharhinus amblyrhyncho* and *Sphyraena **barracuda.*** The physico-chemical characters were studied. The skin of shark yielded higher amount of gelatin than the sheela. Similarly protein, turbidity, pH, Gelling and Melting temperature, Foaming properties, Emulsifying properties, colour, amino acid composition of shark gelatin were in better than the sheela. According to this study, it is discovered that Gelatin can be extracted from fish skin, which is a major waste produced by fish processing industries. In this study, gelatin was extracted from the two fish species shark and sheela. The gelatin was produced by means of extraction process using alkaline and acid pre-treatment followed by extraction with distilled water and drying process. The extracted gelatin exhibited different physicochemical characteristics. The presence of extracted gelatin was confirmed by the gelatinase test and FT-IR analysis. The extracted gelatin was purified by dialysis method. Certain characteristics of extracted gelatin from two different fish skin were evaluated. The protein content of extracted gelatin was found to be 55mgfor Carcharhinus amblyrhyncho and 50mg for Sphyraena barracuda estimated by Lowry's method. The turbidity of the gelatin gradually increased depending on the pH range. The extracted gelatin colour appeared white in Carcharhinus amblyrhyncho and Sphyraena barracuda was yellow in colour. pH of the extracted gelatin was similar to the pH of commercial gelatin. The gelling temperature of the Carcharhinus amblyrhyncho was rapid compared to that of Sphyraena barracuda. The extracted gelatin from both the species were found to exhibit similar melting temperature, Emulsifying stability index of the extracted gelatin increased with

increasing concentration. Foaming properties of the extracted gelatin for Carcharhinus amblyrhyncho was higher than the Sphyraena barracuda.

Samantha (2016) studied the optimization of collagen extraction conditions from fish waste (fish skin, scales, bones and fins) with the aid of response surface methodology (RSM). The statistical optimization method was designed with a 4-factor, 5-level central composite design (CCD), which the effects of acetic acid concentration (M), pH, extraction temperature (°C) and extraction time (h) in response to the extracted collagen yield (%) were fitted into the CCD. Generally, concentration of acetic acid with pH 2.96 to 3.19 at th0e range of 0.56 M to 0.67 M, were able to produce high collagen yields. However, the optimum extraction temperature and extraction time varied among fish waste. Optimum extraction temperature for fish skin was slightly lower (13.26 °C), whereas, scales, bones and fins require higher temperatures (16.6 °C to

19.03 °C). In terms of extraction time, fish scales need longer hours (77.51 h), as compared to

skin (74 h), bones (73.16 h) and fins (72.36 h). The optimized extraction conditions yielded 2.27

% for skin, 0.13 %, 0.64 % and 0.82 % for scales, bones and fins, respectively. SDS-PAGE pattern confirmed that fish skin, scale, bone and fin collagen were all type I collagen consisting of two α1 and α2 chains. Denaturation temperature (*T*d) of collagen from skin, scales, bones and fins were 36 °C, 36.15 °C, 37.80 °C and 32.60 °C, respectively. Besides, fourier transform infrared spectroscopy (FTIR) proved that the collagen of these fish were integrated and native. In addition, reversed-phase high-performance liquid chromatography (RP-HPLC) showed that these collagen had high contents of imino acids, which contributed to their high denaturation temperatures. These findings suggested that fish waste skin, scale, bone and fin collagen possess the potential to be an alternative collagen source for a variety of uses in many fields.

Irwandi *et al.* (2009) investigated the properties in the characterization of gelatin extracted from different marine fish species in Malaysia, Gelatins from the skin of four local marine fish, namely "kerapu" (*Epinephelus sexfasciatus),* "jenahak" (*Lutjianus argentimaculatus),* "kembung" (*Rastrelliger kanagurta),* and "kerisi" (*Pristipomodes typus)* were successfully extracted by acid extraction. The samples were washed with clean sea water at the point of collection, separated by species, and packed in polyethylene plastic bags inside a cooler and transferred to the laboratory. After reaching the laboratory, the samples were stored at -27ºC. Sodium hydroxide, sulphuric acid, and citric acid were purchased from local suppliers. All the chemicals used were of analytical grade. During the extraction, the extraction procedure was conducted according to Grossmane *et al.*, (1992), with slight modifications. The fish were thawed prior to the experiments. The accurately weighed fish were cleaned and washed with tap water followed by peeling the fish skin using a sharp scalpel. The fish skins were

thoroughly rinsed in excess water to remove superfluous materials. The fish skins were soaked in 0.2% (w/v) sodium hydroxide for 40 minutes. After washing out sodium hydroxide, two successive acid incubations were performed, each for 40 min, first in a sulphuric (0.2%, v/v) and then in a citric acid solution (1.0%, w/v). The acid solutions were drained and then samples were washed with cold water once. Results characterization showed that the fish gelatins were comparable to the fish gelatins from other fish species previously reported. They appeared snowy white in color with crystal-like and light texture. The gelatine extracted from "kerapu" had the strongest fishy odor, followed by the gelatines derived from "jenahak", "kembung" and "kerisi". In terms of bloom strength, the gelatin extracted from "kerapu" was found to be the strongest one compared to others, with the bloom value of more than 2000 g. The gelatins developed in this study contained almost all essential amino acids, with glycine being the most predominant one.

Suhair *et al*., (2021) carried out a comprehensive review on the Cosmetic, Biomedical and Pharmaceutical Applications of Fish Gelatin/Hydrolysates**.** Their review covers cosmetic applications, intrinsic activities, and biomedical applications in wound dressing and wound healing, gene therapy, tissue engineering, implants, and bone substitutes. It also covers its pharmaceutical applications including manufacturing of capsules, coating of microparticles/ oils, coating of tablets, stabilization of emulsions and drug delivery (microspheres, nanospheres, scaffolds, microneedles, and hydrogels). The main outcomes that were enumerated in this review are that fish gelatin is immunologically safe, protects from the possibility of transmission of bovine spongiform encephalopathy and foot and mouth diseases, has an economic and environmental benefits, and may be suitable for those that practice religious-based food restrictions, i.e., people of Muslim, Jewish and Hindu faiths. It has unique rheological properties, making it more suitable for certain applications than mammalian gelatins. It can be easily modified to enhance its mechanical properties. However, extensive research is still needed to characterize gelatin hydrolysates, elucidate the Structure Activity Relationship (SAR), and formulate them into dosage forms. Additionally, expansion into cosmetic applications and drug delivery is needed. According to them, fish gelatin extracted from collagen obtained from by-products of the fish industry is by simple methods. It is biocompatible, biodegradable, safe and can be used as another source of gelatin in addition to the mammalian sources (bovine and porcine). A source that is permissible or lawful to Islamic, Jewish, and Hindus laws. Fish gelatin has lower rheological properties and lower melting points than mammalian gelatin. Additionally, cold-water fish gelatin has lower melting point than warm-water fish gelatin. This makes it more suitable for certain applications for example, microencapsulation of oils. On the other hand, it can be easily modified by cross-linking or the use of polymers to enhance its mechanical properties and make it more suitable for other applications,

for example, tissue engineering. Gelatin hydrolysates have many intrinsic activities. However, further studies are needed to elucidate the structure of fish gelatin hydrolysates that are active especially as anticancer and antidiabetic agents, characterize them, elucidate the structure activity relationship and deliver them using suitable routes of administration. These studies are needed to reveal new intrinsic activities against UV radiation. Also, its application in drug delivery is still modest, and expansion in these two fields is needed. For example, the use of films loaded with drug for local delivery to the skin or for systemic drug delivery is a potential application.

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