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Physico-functional and mechanical properties of chitosan and calcium salts incorporated fish gelatin scaffolds

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ABSTRACT

Four types of fish gelatin scaffolds *viz.* gelatin (G), gelatin–chitosan (GC), gelatin–calcium acetate (GCA) and gelatin–chitosan–calcium acetate (GCCA) prepared were investigated for their functional properties, biomechanical strength, microstructural changes in relation to biodegradation. GC scaffold was superior with pH 3.15 and viscosity 9.40 cP. Chitosan and calcium acetate improved tensile strength (TS) and Young's modulus (YM), but lowered elongation at break (EAB). GCCA scaffold possessed moderate TS of 19.6 MPa, EAB of 4.76% and YM of 185 MPa. Foaming ability ratio of GC scaffold was high (3.41). GCA and GCCA scaffolds remained for 4 days before complete *in vitro* biodegradation. GC scaffold had larger cavities (180–300 μm) that were responsible for low swelling ratios and shrinkage factor. GCCA scaffold with moderate swelling rates, mechanical, functional properties and lowered biodegradation rate were found more suitable for biomedical applications.

1. Introduction

Gelatin has been traditionally produced through hydrolysis of skin and bone collagen of bovine or porcine by acidic or alkaline processes. Use of animal gelatin has been restricted in many countries for religious issues as well as disease outbreaks such as bovine spongiform encephalopathy (BSE), mad cow disease, etc. [1]. Hence, fish gelatin has gained prominence as an alternative to animal gelatin. It can be produced from the solid wastes of the fish processing industries such as skin, bones, fins, scales and swimbladder. Extraction of fish gelatin from these wastes has been reported by several workers [2–4]. It has been recently used in the preparation of biodegradable biomedical products such as films, sheets, microspheres and even scaffolds [5].

Biodegradable scaffolds have been constructed from a variety of synthetic polymers such as poly glycolate, poly lactate, their copolymer poly DL-lactic-co-glycolate as well as naturally derived polymers such as gelatin, collagen and chitosan [6,7]. Scaffolds have been formed using gelatin extracted from pig tendon [8], white rabbit's bones [9] and porcine skin [10,11]. Few attempts have been made to form fish collagen scaffolds by few authors using tropical fishes [12], salmon [3] and jellyfish [13]. However, scaffolds have not been formed using fish gelatin. Ogawa et al. [14] opined that the development of fish gelatin/collagen scaffolds would help to utilize the fish processing wastes.

Scaffolds made of mammalian/fish collagen do have certain limitations for biomedical applications compared to those made from other materials. They are low in stiffness and rapid in biodegradation. Song et al. [13] noticed high rate of enzymatic degradation of natural collagen in *in vivo* studies and suggested suitable stabilization methods for collagen based materials. Chitosan biomolecules and calcium acetate salts are expected to stabilize gelatin or collagen based scaffolds, as they possess unique properties to minimize biodegradation. Chitosan, being a linear polysaccharide, comprises β1- to β4-linked D-glucosamine residues. This primary monomer has a similar framework as that of glycosaminoglycans (GAGs) of the cartilage and found responsible for cartilage repair [15]. In addition, chitosan also activates cellular reconstruction and regeneration, stops hemorrhaging and provides anesthetic properties. Calcium salt too plays an important role as coagulation factor and in bone regeneration along with phosphorus. Calcium as a cofactor of enzymes plays an essential role in wound healing process. In addition, it supports normal nervous and muscular activities.

Chirita [16] reported that the addition of chitosan and calcium acetate had improved the mechanical properties of ox skin collagen films, but there is no evidence on their effects on the properties of collagen or gelatin scaffolds. Lin et al. [15] reported that the three factors that affect the rate of *in vitro* biodegradation of scaffolds are the morphology, protection by glycosaminoglycans and chemical cross-linkage. Therefore, in this study, fish gelatin scaffolds were formed with two important biomaterials *viz.* chitosan

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and calcium acetate salts and their functional properties, biomechanical strength and microstructural changes were examined, in relation to their biodegradation rates.

2. Materials and methods

2.1. Raw materials

Skins of catfish (*Pungaseous fungaseous*) were used as raw materials for the extraction of fish gelatin. Skins were obtained from a private fish processing plant, M/s. Britto Seafoods Pvt. Ltd., Tuticorin, South India. They were washed with potable water and cut into small pieces using sharp knives prior to the extraction.

2.2. Extraction of gelatin

Gelatin was extracted as per our earlier reported method [2]. Briefly, skins were first rinsed with tap water to remove superfluous material and then treated twice with 0.2% NaOH at the ratio of 1:6 w/v for 45 min to remove the non-collageneous protein. After thorough washing, they were then treated twice with 0.2% H_2SO_4 at the ratio of 1:6 w/v for 45 min to increase swelling as well as to remove the salts. They were then treated with 1% citric acid twice at the ratio of 1:6 w/v for 45 min to achieve the lowest degree of turbidity and decalcification. The final extraction was carried out with distilled water at the ratio of 1:1 w/v at 45 °C for 24 h. The extract was then filtered through vacuum filter and the filtrate was lyophilized using Lyophilizer (Alpha 2, Martin Christ, Germany).

2.3. Gelatin scaffolds preparation

Four types of gelatin scaffolds were prepared *viz.* simple gelatin (G), gelatin–chitosan (GC), gelatin–calcium acetate (GCA) and gelatin–chitosan–calcium acetate (GCCA). Simple gelatin scaffolds were prepared by dissolving 3% gelatin along with 0.2% glutaraldehyde in 10 ml distilled water. GC scaffolds were prepared with the addition of 1% chitosan (95% degree of deacetylation, Himedia, India), previously dissolved in 0.3 M acetic acid and heated for 20 min after adding to the gelatin solution. GCA scaffolds were prepared with the addition of 1% calcium acetate (Merck, Chennai, India) to the gelatin solution. GCCA scaffolds were prepared with the addition of 1% chitosan and 1% calcium acetate to the gelatin solution. The scaffold forming solution was stirred in a magnetic stirrer for 1 h to produce foam, and was then lyophilized in a lyophilizer (Alpha 2, Martin Christ, Germany) and their physicofunctional and mechanical properties were examined.

2.4. pH and viscosity

The pHs of the gelatin scaffolds forming solutions were measured in a pH meter (Krishna Scientific Supplies, Chennai, India) at 25 ± 0.5 °C according to the procedure of Cheow et al. [17]. The viscosity of gelatin scaffolds was measured as per the method of Leffler and Müller [18]. Gelatin solution, 50 mL was used for the viscosity (cP) measurement in the Brookfield Digital Viscometer (Model DV E, Brookfield Eng Lab Inc., Middleboro, MA) equipped with a No. 1 Spindle at 60 rpm at 30 ± 0.5 °C.

2.5. Mechanical properties

Mechanical properties such as tensile strength (TS), elongation at break (EAB) and Young's modulus (YM) were determined for the gelatin scaffolds by standard ASTM D 882 methods [19] using a Universal Testing Machine (TA plus Texture analyzer, Lloyd instruments, U.K.). The scaffolds were cut into rectangles of size 15 mm × 40 mm and fixed on the grips of the device. They were then pulled apart at crosshead speed of 5 mm min^{-1}. The TS was calculated by dividing the maximum force at break by cross-sectional area of film, and expressed in megapascal. The EAB and YM were calculated based on the maximum length extended at rupture and ratio of stress to strain of the films; expressed in percent and megapascal, respectively. The thicknesses of the gelatin scaffolds were measured to the nearest 5 μm with the Laboratory Micrometer screw gauge (Labtech International, Ambala, Haryana, India) at six random positions.

2.6. Foam formation ability and foam stability

Foam formation ability (FA) and foam stability (FS) of gelatin scaffolds were determined by the procedure of Cho et al. [20]. Gelatin scaffold forming solutions, 25 mL, was taken in a beaker and foam was prepared by stirring the solutions continuously using a magnetic stirrer for 30 min The solution was then poured into a 100 ml measuring jar. The FA and FS rates were calculated by the following formula:

$$\text{foam formation ability (FA)} = \frac{\text{volume of foam (ml)} + \text{volume of liquid (ml)}}{\text{initial volume of solution (ml)}}$$

$$\text{foam stabilty (FS)} = \frac{\text{volume of liquid (initial)}}{\text{volume of liquid (after 30 min)}}$$

2.7. Swelling property (water binding capacity)

Swelling property of the gelatin scaffolds was examined according to the method of Pan et al. [9]. The scaffolds were placed in a phosphate buffered saline (PBS), pH 7.4 at 37 °C. The initial dry weight of the scaffold was noted as W_1. They were then placed in PBS buffer solution at 37 °C for 1 h. The surface adsorbed water was removed by filter paper and the wet weight was recorded W_2. The ratio of swelling was determined using the following equation:

$$\text{swelling ratio (\%)} = \frac{W_2 - W_1}{W_1} \times 100$$

where W_2 is the weight of the wet scaffolds and W_1 is the initial weight of the scaffolds.

2.8. Shrinkage factor

The shrinkage factor of the gelatin scaffolds was determined based on US patent method [8]. The scaffold weighing 1.0 g was placed in 10 ml distilled water at 37 °C for 15 min and its surface area was measured. It was then lyophilized for 7 h in a lyophilizer and its final surface area was measured. The shrinkage factor was arrived from the difference between the area values obtained before and after lyophilization.

$$\text{shrinkage factor (\%)} = \frac{(A_b - A_a)}{A_b} \times 100$$

where A_b is the surface area of the scaffold before lyophilization and A_a is the surface area of scaffold after lyophilization.

2.9. In vitro biodegradation

In vitro biodegradation of gelatin scaffolds was determined as per the procedure of Mao et al. [21]. The scaffolds (0.1 g) were incubated with 1 ml of PBS (pH 7.4) at 37 °C containing 1.6 μg/ml trypsin (SISCO Research Laboratory Pvt. Ltd., Mumbai, India) until complete biodegradation. The enzyme solution was refreshed daily to ensure continuous enzymatic activity. Samples were taken daily from the