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Research Article

The Characterization of Fish (Tilapia) Collagen Sponge as a Biomaterial

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For scaffold manufacturing, the utility of bioactive natural organic materials derived from marine products is useful and indispensable as an alternative to bovine collagen. The weakest feature of fish collagen for scaffold application is its low degeneration temperature (T_d), indicating poor stability of fish collagen in mammals *in vivo*. We have focused on the tropical fish tilapia as a candidate for generating a clinical scaffold. The aim of this study was to confirm the T_d of tilapia type I atelocollagen (TAC) for biomedical application. Furthermore, the physical and structural properties were investigated and evaluated as a scaffold on a sponge form. Different concentrations {0.5%, 1.0%, and 2.0% (v/v)} of TAC solution were analyzed. Differential scanning calorimetry showed that the T_d of TAC was 35-36°C. The scanning electron microscopy results indicated that the pore size (90–160 μm) of TAC sponges is acceptable for cell proliferation. The tensile strength of porous sponges was in the range of 0.01–0.07 MPa. These findings indicate that the TAC sponge prepared from tilapia is one of candidates as a scaffold. The 1.0% (v/v) concentration of TAC solution is especially recommended to be advantageous for preparing and handling the solution and for sponge formation.

1. Introduction

Regenerative medicine consists of three components: cells, nutrients, and scaffold [1]. For scaffold manufacturing in regenerative medicine, the utility of bioactive natural organic materials originating from marine products is useful and indispensable, because severe infections (zoonosis) such as bovine spongiform encephalopathy, avian and swine influenza, tooth-and-mouth disease in cows, pigs, and buffalos, and Ebola hemorrhagic fever (transmitted by infected bats) occur worldwide. According to this background, fish (the most genetically distant from mammals) collagen derived from scales, skin, and bone has been of strong interest to laboratories worldwide for its potential application as a scaffold biomaterial and carrier, due to its bioactivity properties, such as excellent biocompatibility, low antigenicity, and high biodegradability and cell proliferation potential [2, 3].

Several previous studies have demonstrated that the amino acid composition of fish collagen is similar to that of mammalian collagen [4]. For example, glycine is the most abundant amino acid, accounting for more than 30% of all amino acids. The weakest feature of fish collagen as a scaffold is its generally low degeneration temperature (T_d), thus indicating the poor stability of FC in mammals *in vivo*. The lack of stability of FC is thought to be due to its low hydroxyproline content compared with that observed in bovine collagen [5]. Recently, we have focused on the tropical fish tilapia as a powerful candidate generating a clinical scaffold, as an alternative to bovine collagen, and have confirmed the biological safety of tilapia type I atelocollagen (TAC) in accordance with ISO standards [6].

The aim of this study was to first confirm the T_d of TAC for biomedical application. Furthermore, its physical and structural properties were investigated and evaluated on a sponge

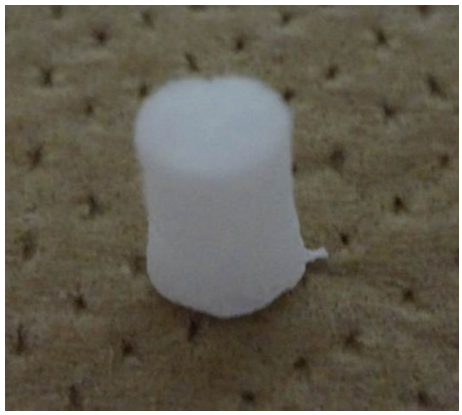


Figure 1: Freeze-dried sponge sample.

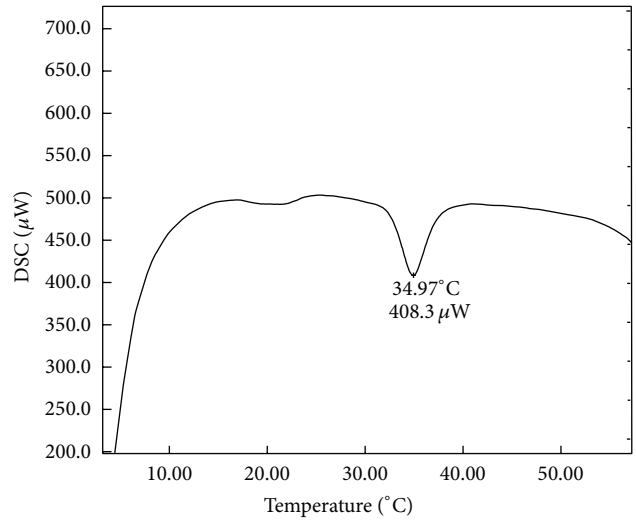


Figure 2: The typical DSC spectrum of the 1% concentration of TAC solution. Note that there are no peaks of T_g and T_c. The valley corresponding to T_m presents T_d.

form, acting as a scaffold, with different concentrations of TAC solution used for the physicochemical analyses.

2. Materials and Methods

2.1. Preparation of the TAC Solution. A 5% (v/v) solution of fish collagen derived from tilapia skin in 5 mM acetic acid was kindly supplied by Nippi Inc., Biomatrix Institute (Ibaragi, Japan). TAC solutions of 0.5%, 1%, or 2% (v/v) concentrations were prepared by dissolving the 5% (v/v) TAC solution in 5 mM acetic acid. This solution was stirred gently for 2~5 h at 50°C until it became transparent. Thereafter, it was neutralized to pH 7.4 using 1 N NaOH and then filtered using a 0.45 μm filter. Furthermore, the air remaining in the solution was removed by a vacuum pump for 24 h.

2.2. Differential Scanning Calorimetry (DSC) Analysis. The T_d of the above-mentioned fish collagen solution was detected using a differential scanning calorimeter (EXSTAR DSC6000; Hitachi High-Tech Science Corp., Tokyo, Japan) with a constant heating rate of 1°C/min over the range of 0 to 60°C. The samples were thoroughly degassed prior to each experiment. Measurements were performed in three experiments for each TAC concentration.

2.3. Sponge Form Fabrication. TAC scaffolds were fabricated using a thermally induced phase separation method. Bulk TAC sponge samples were prepared by freezing and lyophilizing TAC solutions in a prechilled 96-well plate. The final cylindrical sample was 5 mm in diameter and 10 mm in height (Figure 1), accomplished using 0.4 mL/well of the above solution and followed by refrigeration at 4°C for 2 h. The subsequent cooling regimen was performed at 0°C for 18 h and −35°C for 24 h, followed by −80°C for 24 h. Finally, the solution was completely lyophilized by freeze-drying at −80°C for 72 h [7].

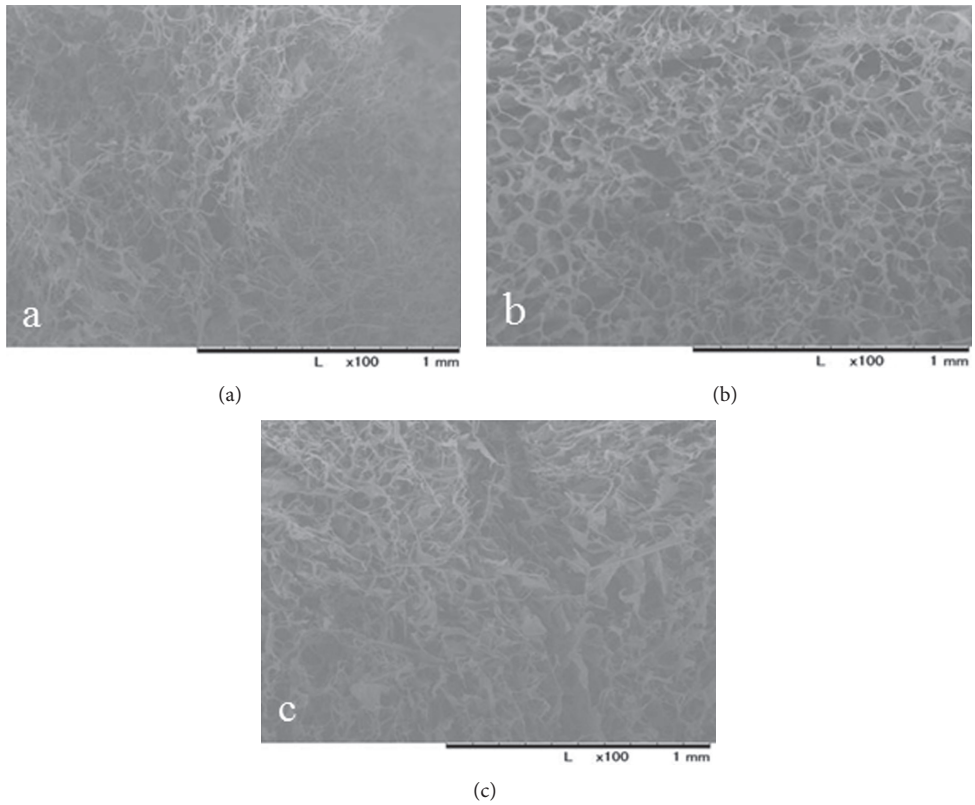
2.4. Microstructural Characteristics. To observe the surface and internal microstructure of the fish collagen sponge, the freeze-dried samples were mounted on aluminum holders and coated with carbon using a vacuum evaporator. Their pore structures were examined using a Hitachi S-3500 scanning electron microscope (Hitachi Ltd., Tokyo, Japan) operated at 20 kV. The mean pore diameters were estimated from scanning electron micrographs by measuring 50 different pores in each scaffold using a computed image analyzer [8]. A comparative study on the pore diameter was performed in the sponges prepared from the different concentrations of FC solution.

2.5. Mechanical Properties. The ultimate tensile strength of the scaffold in a wet state was measured using a universal testing machine (AG-X 50N, SHIMAZU, Kyoto, Japan) according to the procedures outlined in the ASTMD 3574-E at room temperature. The three-dimensional cylindrical scaffold was thoroughly hydrated using a 0.1 M PBS solution while mounted on the grip, and then it was pulled until it broke with a constant crosshead speed of 1 mm/min under 65% relative humidity until failure [9]. A tensile stress-strain curve was generated from each sample, and Young's modulus was calculated by drawing a tangent to the initial linear portion of the stress-strain curve. The measurements were performed on five samples.

2.6. Statistical Analysis. The statistical significance ($P < 0.05$) of the differences between the two groups was assessed using paired Student's t-test. All values were expressed as the means ± standard deviations (SD).

3. Results

3.1. DSC Analysis (Table 1). The typical differential spectrum presented a single valley (T_d), which indicated the melting temperature (T_m) (Figure 2). Table 1 shows the average T_d at the different concentrations of TAC solution. There were no



(a) (b) (c)

Figure 3: The surface images of sponges produced from different concentrations of TAC solution: (a) 0.5% (v/v), (b) 1.0% (v/v), and (c) 2.0% (v/v).

Table 1: The T_d of sponge with different concentrations of TAC.

Type of sponge	T_d
0.5% (v/v)	35.3 ± 0.34
1.0% (v/v)	35.50 ± 1.00
2.0% (v/v)	34.68 ± 0.49

Table 2: The pore sizes of sponge with different concentrations of TAC.

Type of sponge	Average diameter (μm)
0.5% (v/v)	158.3 ± 31.4
1.0% (v/v)	112.5 ± 26.9
2.0% (v/v)	94.5 ± 25.3

significant differences in the T_d among the three different concentrations of TAC solution ($P > 0.05$).

3.2. Microstructural Characteristics (Figure 3). The surfaces of each sponge with interconnected pores appeared to be similar among the different concentrations of TAC solution. Table 2 shows the average diameter of the pores of three different TAC sponge concentrations. There were significant differences in the pore size between the 2% and the other two sponge concentrations ($P < 0.05$). Although there was no significant difference between the 0.5% and 1.0% sponges, the pore size of 1.0% sponge had smaller tendency than that of the 0.5%.

Table 3: The mechanical properties of sponge with different concentrations of TAC under wet conditions.

Type of sponge	Tensile strength (MPa)	Young's modulus (MPa)
0.5% (v/v)	0.01 ± 0.01	0.01 ± 0.01
1.0% (v/v)	0.04 ± 0.02	0.13 ± 0.03
2.0% (v/v)	0.07 ± 0.01	0.62 ± 0.08

3.3. Mechanical Properties (Table 3). Table 3 shows the mechanical properties (tensile strength and Young's modulus) under wet conditions of the three different TAC sponge concentrations, among which there was a significant difference ($P < 0.05$).

4. Discussion

DSC analyses were performed to investigate the thermostability of TAC. The T_d is important for the clinical application of biomaterials as scaffolds in regenerative medicine. Since the T_d of fish collagen is lower than the body temperature of mammals, fish collagen melts when placed in contact with the human body during clinical application. Recently,

interesting data have indicated that collagen extracts derived from the scales of tropical fish (tilapia) have a T_d of 35°C [10]. This high level of T_d observed in tropical fish is highly consistent with the high degree (e.g., tilapia: 43%) of hydroxylation of proline in collagen among warm seawater fish [11], compared with that of cold seawater fish such as chum salmon (35–37%) [12, 13]. These findings together with the present T_d data (35-36°C) suggest that tilapia collagen may be used in human medical applications at actual physical temperatures and that tilapia collagen may be suitable for proper biodegradation *in vivo* (our unpublished preliminary rat data that this sponge disappeared within 1 week after implantation), since this relatively high T_d is obtained without the need for chemical cross-linking. Furthermore, the tilapia collagen described here was purified from solubilized tilapia skin, which is thought to be profitable for obtaining a stable supply, compared with scales.

The pore size of scaffolds may be regulated by altering and controlling the freezing temperature. Furthermore, it was revealed that the higher the concentration of the TAC solution, the thicker (the smaller pore size) and more homogenous the pore wall. This finding suggests that the viscosity increased with higher concentrations of TAC solution. There are no significant differences in cell infiltration into sponges with pore sizes ranging from 50 to 200 μm [14]. The present data indicate that the pore size of TAC sponges is acceptable for cell seeding and cell proliferation.

The data on mechanical properties differed completely from those on pore size, in relation to the concentration of TAC. The higher the concentration of TAC, the greater the values of tensile strength and Young's modulus. These findings indicate that a high concentration of TAC makes it possible to create a dense and thick pore wall, which results in improved mechanical properties. The tensile strength of porous structures is generally in the range of 0.03–0.06 MPa [15, 16]. The pore size also affects the mechanical properties of porous scaffolds, in which the spaces with smaller pore sizes of poly(L-lactic acid) (PLLA) have weaker compressive mechanical properties compared with larger pore sizes [17, 18]. These previous findings indicate that the property of a compressive strength in PLLA on pore sizes is different from that of the present tensile strength in TAC and that the 1% or 2% (v/v) concentration of TAC sponge is acceptable, based on the mechanical property criteria for scaffold material.

5. Conclusion

Type I atelocollagen sponge prepared from tilapia skin becomes one of the candidates as a scaffold in regenerative medicine due to its physical and structural properties. The sponge obtained from a 1.0% (v/v) concentration of TAC is therefore considered to be advantageous for both preparing and handling the TAC solution and for sponge formation.

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors' Contribution

The first three authors contributed equally to this work.

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