CHAPTER 3

METERIALS AND METHODS

3.1 Materials

Nile tilapia (*Oreochromis niloticus)* skin, a by-product of the fish processing industry, was obtained from Xian-Ning Seafood Co., Ltd., Phetchaburi, Thailand. The skins were placed in salt during transportation from Phetchaburi to Bangkok. Upon arrival at the Department of Food Technology, Chulalongkorn University, fish skins were washed with tap water. The remaining meat and scales were removed from the skin. Skins were stored at -20°C until further used. Descaled skin was subjected to proximate analysis according to AOAC's method (2000).

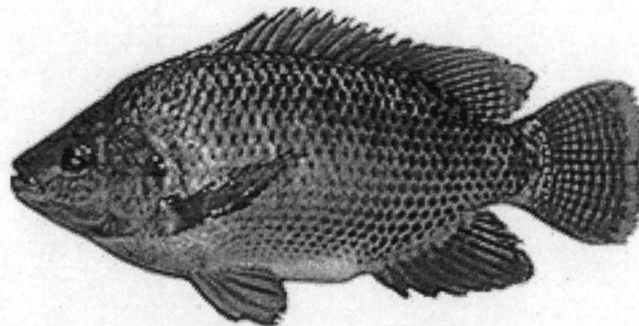

Figure 3.1 Nile tilapia (*Oreochromis niloticus)*.

3.2 Chemical reagents

<u>Chemicals for proximate composition determination</u>

Sulfuric acid (J.T. Baker, USA)	(A. R. grade)
Boric acid (Univa, Ajax Finechem, Australia)	(A. R. grade)
Selenium reagent mixture (Merck, Darmstadt, Germany)	(A. R. grade)
Sodium hydroxide (Univa, Ajax Finechem, Australia)	(A. R. grade)

Methylene blue (Merck, Darmstadt, Germany)	(A. R. grade)
Petroleum ether (Carlo Erba Reagenti, Rodano, Italy)	(A. R. grade)
Potassium hydrogen phthalate (Univa, Ajax Finechem, Australia)	(A. R. grade)
Methyl red (Merck, Darmstadt, Germany)	(A. R. grade)
Hydrochloric acid (J.T. Baker, USA)	(A. R. grade)

Chemicals for gelatin extraction

Sodium hydroxide (Univa, Ajax Finechem, Australia)	(A. R. grade)
Hydrochloric acid (J.T. Baker, USA)	(A. R. grade)

Chemicals for protein pattern determination

Sodiumdodecyl sulphate (Bio-Rad Laboratories, CA, USA)	(A. R. grade)
Tris-HCl buffer (Bio-Rad Laboratories, CA, USA)	(A. R. grade)
Ammonium persulfate (Bio-Rad Laboratories, CA, USA)	(A. R. grade)
TEMED (Bio-Rad Laboratories, CA, USA)	(A. R. grade)
β- mercaptoethanol (Bio-Rad Laboratories, CA, USA)	(A. R. grade)
Acrylamide (Bio-Rad Laboratories, CA, USA)	(A. R. grade)
Bisacrylamide (Bio-Rad Laboratories, CA, USA)	(A. R. grade)
Coomassie blue R-250 (Bio-Rad Laboratories, CA, USA)	(A. R. grade)
Glycine (Sigma-aldrich, USA)	(A. R. grade)
Glycerol (Merck, Darmstadt, Germany)	(A. R. grade)
Methanol (Sigma-aldrich, USA)	(A. R. grade)
Acetic acid (Sigma-aldrich, USA)	(A. R. grade)
Bromophenol blue (Bio-Rad Laboratories, CA, USA)	(A. R. grade)

Chemicals for electrospinning process

Acetic acid (Sigma-aldrich, USA)	(A. R. grade)
Formic acid (Sigma-aldrich, USA)	(A. R. grade)

Glutaraldehyde (Sigma-aldrich, USA) (A. R. grade)

3.3 Instruments

1. High voltage power supply (Model D-ES30PN/M692, Gamma high voltage research, Florida, USA)
2. Viscometer (Brookfield DV-III, Brookfield Engineering Laboratories, MA, USA)
3. Conductivity meter (Orion 160, Orion Research Inc., Beverly, MA, U.S.A)
4. Chroma Meter (Model CR-300 Series, Minolta, Japan)
5. Scanning electron microscope (JEOL model JSM-6400 LV, Peabody, MA, USA)
6. Ion sputtering (JEOL JFC-1100E, Peabody, MA, USA)
7. Rotary vacuum evaporator (EYELA N-N series, Tokyorikakikai Co., LTD, Tokyo, Japan)
8. Hot air oven (Model 600, Memmert, oxford, Connecticut, USA)
9. Muffle furnaces (Fisher scientific Model Isotemp, USA)
10. Distillation unit (BÜCHI B-324, BÜCHI Labortechnik AG, Flawil, Switzerland)
11. Digestion unit (BÜCHI K-424, BÜCHI Labortechnik AG, Flawil, Switzerland)
12. Soxhlet (Gerhardt, EV-16, königswinter, Germany)
13. Water bath (TECHNE, TE-10D, Tempunit, Cambridge, UK)
14. Universal testing machine (Instron 5565, Canton, MA, USA)
15. Universal testing machine (Lloyd Model LRX, Fareham, Hans, UK)
16. Bohlin Rheometer (C-VOR, Malvern Instruments Ltd., UK)
17. High performance liquid chromatography (Agilent 1100 series, Agilent Technologies, Inc., CA, USA)
18. Evaporative light scattering detector (Alltech ELSD 2000ES, Grace Davison Discovery Sciences Headquarters, Illinois, USA)
19. pH meter (CyberScan pH 1000, Eutech Instruments, Singapore)
20. Electrophoresis apparatus (Mini-Protean II, Bio-Rad Laboratories, Richmond, CA, USA)

3.4 Extraction of gelatin from nile tilapia skin

The gelatins was extracted from fish skin by the method of Ockerman and Hansen (1988) with a slight modification as described in the following scheme (Figure 3.2).

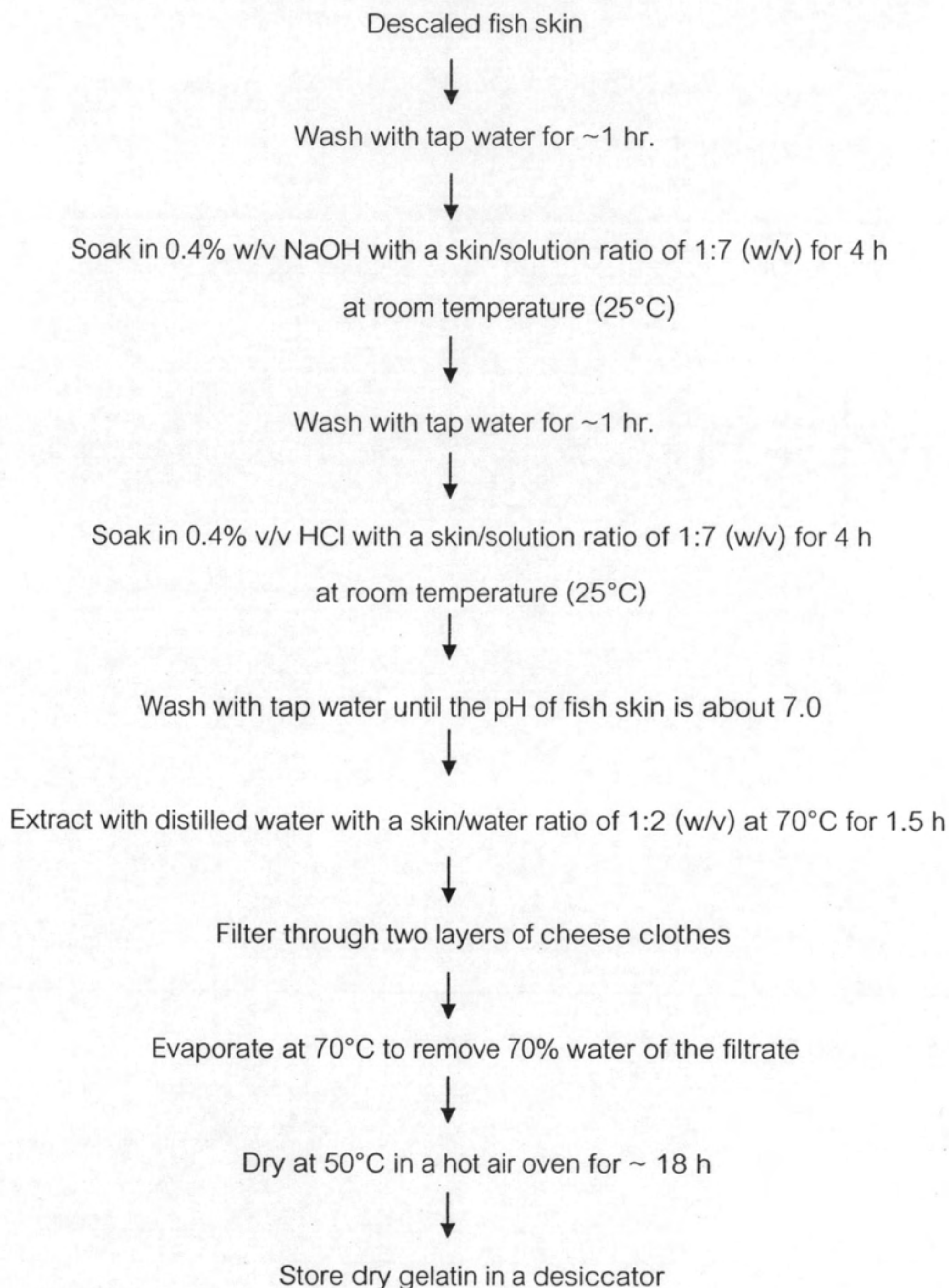

Figure 3.2 Extraction of gelatin from nile tilapia skin.

3.5 Characterization and physical properties of gelatin from nile tilapia skin

3.5.1 Determination of chemical compositions

Moisture, ash, fat and protein contents of gelatin were determined according to the method of AOAC (2000).

3.5.2 Determination of gelatin gel strength

Bloom gel strength was determined according to the British Standard 757: 1975 method (British Standards Institution, 1975) with a slight modification. Gelatin (7.5 g) was mixed with 105 mL of distilled water in a bloom bottle to obtain a final concentration of 6.67% (w/v). The mixture was left at room temperature for 15 min-2 hours to allow the gelatin to absorb water and swell. After this time, the mixture was heated at 65 °C on a magnetic heater stirrer for 20 min to dissolve the gelatin completely. The beakers were then kept in a refrigerator at 10 °C and matured for 16-18 hours. Bloom gel strength at 10 °C was determined by a universal testing machine (Instron 5565, Canton, MA, USA) using a 5 kN load cell equipped with a 1.27 cm diameter flat-faced cylindrical Teflons plunger. The maximum force (in grams), taken when the penetration distance of 4 mm was obtained, was recorded. The speed of the plunger was 0.5 mm/s. The measurement was performed in triplicate.

3.5.3 Viscosity of gelatin solution

The viscosity was determined according to the British Standard 757: 1975 method (British Standards Institution, 1975) with a slight modification. The 6.67% (w/v) gelatin solution at 60 °C was measured by a Bohlin rheometer (C-VOR, Malvern Instruments Ltd., UK) using a cone and plate geometry (cone angle 4°, gap=0.15 mm). The shear rate was 80 s^{-1}. The measurement was carried out in triplicate.

3.5.4 Colour of gelatin powder

The colour of gelatin powder was determined using a chroma meter (model CR-300 Series, Minolta, Japan). CIE L*, a* and b* values were measured.

3.5.5 pH of gelatin solution

The pH of gelatin solution was determined according to the British Standard 757: 1975 method (British Standards Institution, 1975). Gelatin (1 g) was mixed with heated distilled water to dissolve the gelatin completely. The mixture was added with 105 mL of distilled water in a bloom bottle to obtain a final volume of 100 mL. It was left to cool at room temperature. The pH was measured using a pH meter (CyberScan pH 1000, Eutech Instruments, Singapore)

3.5.6 Amino acid analysis

The amino acid composition was determined by the method of Petritis, Elfakir and Dreux (2002) with a slight modification. A 10 mg sample was digested for 24 h in 5 mL of 6 N HCl heated at 110 °C. The hydrolyzate was dried by N_2 stream evaporation. The sample was dissolved and made to volume by deionized water pH 7.0. Then, the sample was filtered and determined by a high performance liquid chromatography (Agilent 1100 series, Agilent Technologies, Inc., CA, USA) equipped with an evaporative light scattering detector (Alltech ELSD 2000ES, Grace Davison Discovery Sciences Headquarters, Illinois, USA) and a reversed-phase column (Prevail C18 Part NO. 99210, Grace Davison Discovery Sciences Headquarters, Illinois, USA) which had a diameter of 4.6 mm and 250 mm long. 5 mM NFPA in 0.7% TFA and acetonitrile was used as the mobile phase with a flow rate of 1 mL/min. N_2 flow rate was 1.5 L/min.

3.5.7 SDS- polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed by the method of Laemmli (1970) with a slight modification. Gelatin was dissolved at 4 mg/mL in distilled water at 60 °C and filtered

through Whatman paper No. 1. Solubilized samples were mixed with the sample buffer (0.5 M Tris-HCL, pH 6.8 containing SDS, glycerol) in presence of β-mercaptoethanol. Samples were loaded into polyacrylamide gel made of 7.5% separating gel and 4% stacking gel subjected to electrophoresis at 200 V using Mini-Protean II unit (Bio-Rad Laboratories, Richmond, CA, USA). After electrophoresis, the protein bands were stained with Coomassie brilliant blue R-250. Gelatin from porcine skin was purchased from Ajax Finechem, Australia. Broad range protein molecular weights marker was used as a standard (Bio-Rad Laboratories, Richmond, CA, USA).

3.6 Production of nile tilapia skin gelatin nanofiber by electrospinning process

3.6.1 Determination of solvent concentration

This part deals with the determination of solvent concentration that is suitable for the production of gelatin nanofibers in single solvent system. Figure 3.3 shows the process for electrospinning of gelatin nanofibers.

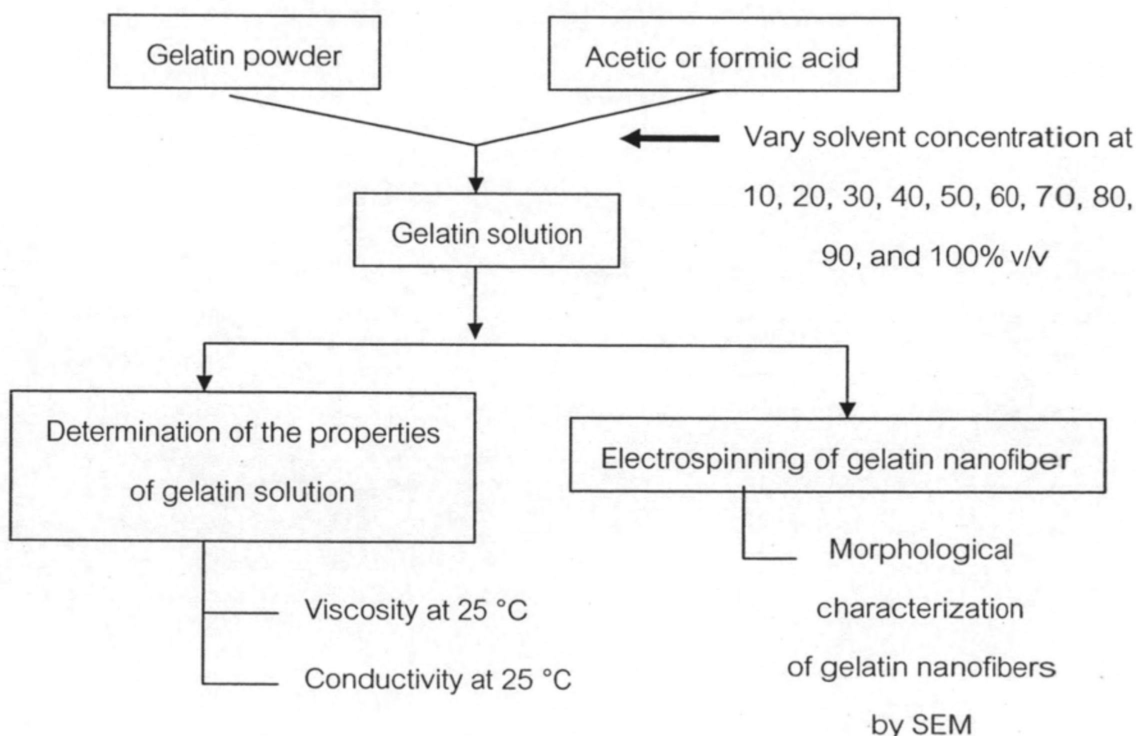

Figure 3.3 Flow chart of electrospinning process for single solvent system.

3.6.1.1 Preparation of gelatin solution

Gelatin solution was prepared by dissolving fish skin gelatin powder in acetic or formic acid. The concentrations of acetic or formic acid were 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100% v/v. The fish skin gelatin concentration was fixed at 15% w/v. The gelatin solution was stirred for 4 hours at 40 °C in order to obtain homogeneous solution.

3.6.1.2 Electrospinning process

An electrospinning apparatus was setup as shown in Figure 3.4. The solution was loaded in a 5 mL glass syringe. The stainless steel tip of syringe was connected to a high voltage power supply (Model D-ES30PN/M692, Gamma high voltage research, Florida, USA). A grounded counter electrode was connected to aluminium foil which was used as a fiber collecting target. The applied electrostatic potential used was 15 kV and the distance between the syringe tip and the grounded target was 15 cm. The spinning time was 10 minutes.

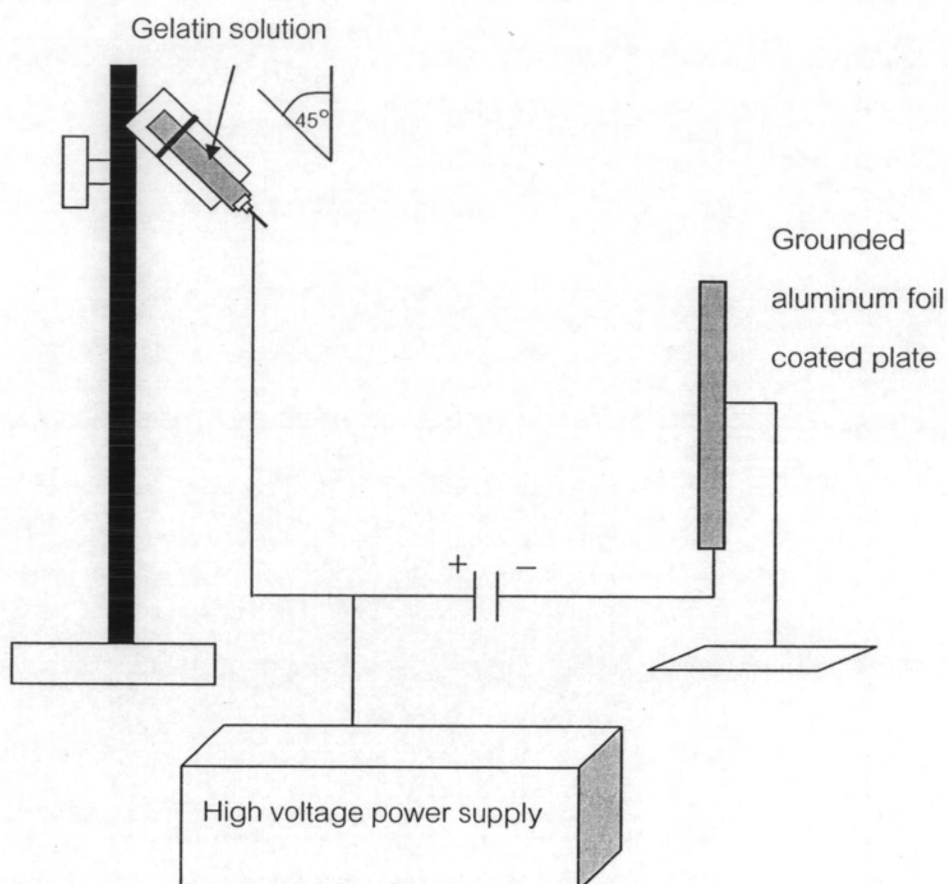

Figure 3.4 Setup of the electrospinning apparatus for the production gelatin nanofibers.

3.6.1.3 Characterization of gelatin solution

The viscosity of gelatin solutions were measured using a Brookfield viscometer (model DV-III, Brookfield Engineering Laboratories, MA, USA) with spindle No. 21 and speed of 10 rpm at 25 °C. The conductivity of gelatin solution was determined by a conductivity meter (model Orion 160, Orion Research Inc., Beverly, MA, U.S.A). The solutions temperature was adjusted prior to the measurements to 25 °C. All measurements were carried out in triplicate.

3.6.1.4 Characterization of gelatin nanofibers

The gelatin nanofiber mat was coated with gold by an Ion sputtering (JEOL JFC-1100E, Peabody, MA, USA) at 10 mA for 4 minutes. The morphology of the

mat was characterized by a scanning electron microscope (JEOL model JSM-6400 LV, Peabody, MA, USA). Nanofiber diameter was analyzed using an image analyzer software (Image J, JEOL, USA)

3.6.2 Determination of gelatin concentration

This part deals with the determination of gelatin concentration that is suitable for the production of gelatin nanofibers in single solvent system. Figure 3.5 shows the process for electrospinning of gelatin nanofibers.

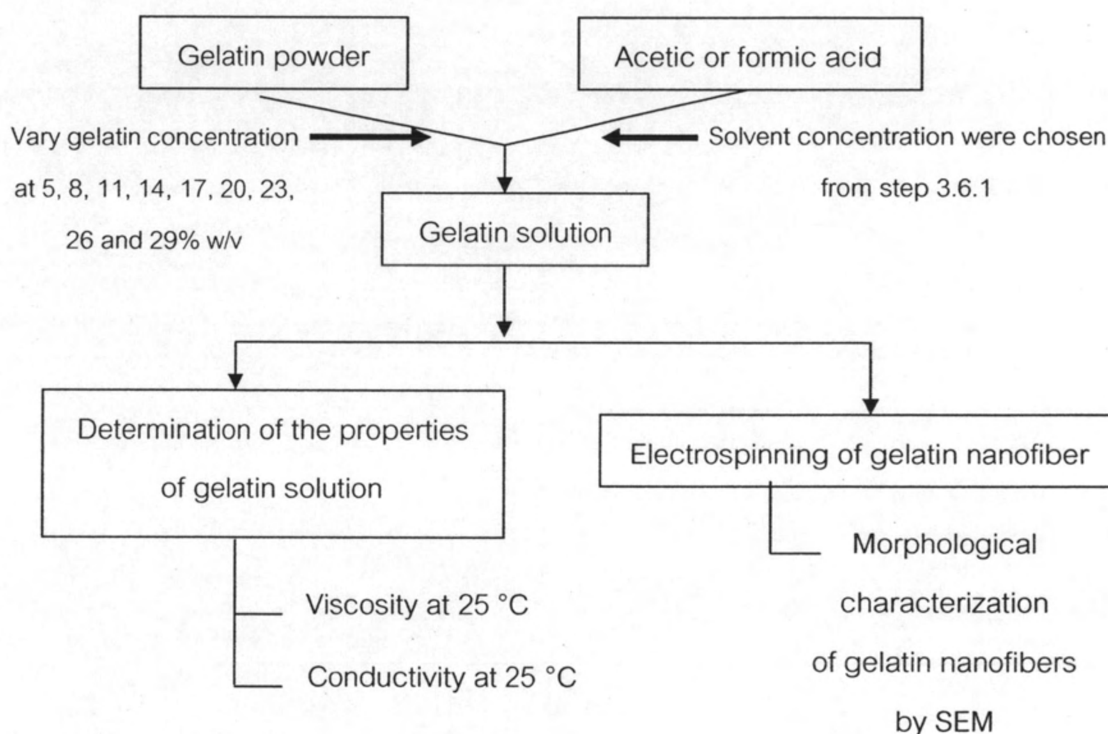

Figure 3.5 Flow chart of electrospinning process with varying gelatin concentrations.

3.6.2.1 Preparation of gelatin solution

Gelatin solution was prepared by dissolving fish skin gelatin powder in acetic or formic acid. The concentrations of acetic or formic acid were chosen from the

previous study. The fish skin gelatin concentrations were varied at 5, 8, 11, 14, 17, 20, 23, 26 and 29% w/v. The gelatin solution was stirred for 4 hours at 40 °C.

3.6.2.2 Electrospinning process

An electrospinning apparatus was setup as shown in Figure 3.4 and as described in section 3.6.1.2.

3.6.2.3 Characterization of gelatin solution

The viscosity and conductivity of the gelatin solutions were measured following the method described in section 3.6.1.3.

3.6.2.4 Characterization of gelatin nanofibers

The morphology of electrospun fibers was evaluated by a scanning electron microscope as described in section 3.6.1.4.

3.6.3 Production of nile tilapia skin gelatin nanofiber mat

3.6.3.1 Preparation of gelatin solution

Gelatin solution was prepared by dissolving fish skin gelatin powder in acetic or formic acid. The concentrations of solvent and gelatin were chosen from step 3.6.1 and step 3.6.2. The gelatin solution was stirred for 4 hours at 40 °C in order to obtain homogeneous solution.

3.6.3.2 Electrospinning process

Preparation of electrospun gelatin nanofibers from a laboratory electrospinning setup has been reported in step 3.6.1.2. A stainless drum was used as the grounded target collector. The applied electrostatic potential used was 15 kV and

the distance between tip and ground target was 15 cm. The spinning time was about 3 days.

3.6.3.3 Crosslinking of gelatin nanofiber mat with glutaraldehyde

The crosslinking process was performed following the method of Zhang *et al.* (2006) with a slight modification. This process was carried out by placing the gelatin nanofibers mat on a supporting stainless steel screen in a glass chamber containing 30 mL of glutaraldehyde solution. The mats were placed on a holed glass shelf in the chamber and were crosslinked by glutaraldehyde vapor at 37 °C for 3 hours. After crosslinking, the mats were placed in a fume hood for 2 hours to allow evaporation of glutaraldehyde followed by a post-treatment by drying in a hot air oven at 100 °C for 1 hour to remove residual glutaraldehyde.

3.6.3.4 Characterization of gelatin nanofiber mat before and after crosslinking

3.6.3.4.1 Morphology of gelatin nanofiber mat

The gelatin nanofibers mat before and after the crosslinking was evaluated by a scanning electron microscope as described in section 3.6.1.4.

3.6.3.4.2 Mechanical properties of gelatin nanofiber mat

The tensile strength, Young's modulus, and elongation of the electrospun gelatin nanofiber mat (width x length = 10 mm x 80 mm) before and after crosslinking were determined using a universal testing machine (Lloyd Model LRX, Fareham, Hans, UK) using a 50 N load cell. A cross-head speed of 10 mm/min was used for all of the specimens tested. The gauge length was 30 mm. The thickness of the mat for the as-electrospun and crosslinked glutaraldehyde fiber mat was around 120-140 μm and 70-95 μm, respectively.

3.6.3.4.3 Colour of gelatin nanofiber mat

The colour of gelatin nanofiber mat before and after crosslinking was determined by a chroma meter values were measured.

3.7 Statistical analysis

All data were subjected to analysis of variance (ANOVA). Comparison of means was carried out by Duncan's multiple range test. Statistical analysis was performed using the Statistical Package for Social Science (SPSS for windows version 10.0, SPSS Inc., Chicago, IL, USA).