CHAPTER III
EXPERIMENTAL

3.1 Materials

3.1.1 Materials for Fabrication of the Neat and the Silver Nanoparticle Loaded Gelatin Hydrogels

Gelatin powder (type A; porcine skin; 170-190 Bloom) was purchased from Fluka (Switzerland). Silver nitrate ($AgNO_3$; 99.998 % purity) was purchased from Fisher Scientific (USA). Glacial acetic acid was purchased from Mallinckrodt Chemicals (USA). Saturated glutaraldehyde aqueous solution (5.6 M or 50 % in water; used as the cross-linking agent) was purchased from Fluka (Switzerland). Sodium metabisulfite powder ($Na_2S_2O_5$) was purchased from Riedel-de Haën (Germany).

3.1.2 Materials for Fabrication of the Neat and the Silver Nanoparticle Loaded Silk Fibroin Hydrogels

Cocoon from *Bombyx mori* silk worm for the preparation of SF was purchased from Phetchabun, Thailand. Silver nitrate ($AgNO_3$; 99.998 % purity) was purchased from Fisher Scientific (USA). Sodium carbonate was purchased from Mallinckrodt Chemicals (USA). Methanol was purchased from Labscan (Asia, Thailand). All other chemicals were of analytical reagent grade and used without further purification.

3.1.3 Materials for Preparation of Acetate Buffer Solution

Sodium acetate and glacial acetic acid were purchased from Ajax Chemicals (Australia) and Carlo Erba (Italy), respectively.

3.1.4 Materials for Preparation of Phosphate Buffer Saline Solution

Sodium chloride, potassium chloride, disodium hydrogen phosphate and potassium dihydrogen phosphate were purchased from Labscan (Asia, Thailand).

3.1.5 Materials for Preparation of Simulated Body Fluid Solution

Sodium chloride, sodium hydrogen carbonate, potassium chloride, dipotassium hydrogen phosphate, magnesium chloride, calcium chloride, sodium sulfate, tris-(hydroxymethyl)aminomethane, hydrochloric acid were purchased from Labscan (Asia, Thailand).

3.1.6 Materials for Cell Culture

Normal human dermal fibroblasts (NHDF) were used as reference cells. Dulbecco's modified Eagle's medium (DMEM; Invitrogen Corp., USA), supplemented by 10 % fetal bovine serum (FBS; Invitrogen Corp., USA), 1 % L-glutamine (Invitrogen Corp., USA) and 1 % antibiotic and antimycotic formulation [containing penicillin G sodium, streptomycin sulfate, and amphotericin B (Invitrogen Corp., USA)]. Saturated glutaraldehyde aqueous solution (5.6 M or 50 % in water was used to fix the cells. Ethanol purchased from Labscan (Asia, Thailand) and hexamethyldisilazane purchased from Sigma-Aldrich (USA) were used to dehydrate the cells.

3.2 Equipment

3.2.1 Equipment for Characterization of Materials

- A JEOL JSM-6400 scanning electron microscope (SEM) was used to observe the morphology of the materials.
- A JEOL JFC-1100E sputtering device was used to coat the materials with a thin layer of gold prior to observation under SEM.
- A Lloyd LRX universal testing machine was used to examine the mechanical integrity of the materials.
- A Shimadzu UV-2550 UV-vis spectrophotometer was used to measure the amount of CM in the sample solutions at wavelength of 300-900 nm.
- A SpectraMax M2 Microplate Reader was used to measure the absorbance of solution from MTT assay at wavelength of 550 nm.

- A Varian SpectrAA-300 atomic absorption spectroscope (AAS) was used to determine the amount of silver released.
- A Thermo Scientific GENESYS 20 4001/4 spectrophotometer was used to measure the optical densities of the bacterial suspensions at wavelength of 600 nm.
- A Burrell Scientific Model AA Wrist-Action® shaker was used to shake the flask that contained the hydrogel absorbing the bacteria.
- A hemocytometer (Hausser Scientific, USA) was used to count the number of the cells.

3.3 Methodology

3.3.1 Preparation of the Sample Solutions

3.3.1.1 Preparation of the Neat and nAg-Containing Gelatin Solutions

$AgNO_3$ at 0.75, 1.0, 1.5, 2.0 and 2.5% by weight of the dry gelatin powder was dissolved in 70 vol.-% acetic acid aqueous solution or warm distilled water. Gelatin powder was then added into the $AgNO_3$ solution and the concentration of gelatin in the solution was 10 wt.-%. The $AgNO_3$-containing gelatin solution was then aged at various time intervals to allow the formation of nAg within the solution.

3.3.1.2 Preparation of the Neat and nAg-Containing Silk Fibroin Solutions

A cocoon was degummed three times with 0.5 wt.- % Na_2CO_3 solution at 95 °C for 30 min to completely eliminate sericin from the fibroin fiber and washed thoroughly several times in hot distilled water and then dried in an oven at 40 °C overnight. Degummed silk as SF fiber at 10 %w/v was dissolved in mixed solvent system of $CaCl_2$: ethanol : H_2O (1 : 2 : 8 in molar ratio) at 78 °C for 1 h to obtain a regenerated SF solution, unlike its native counterpart, which is fully amorphous, with its chains adopting a random-coil, rather than crystallization part. The regenerated SF solution was filtered with a filter paper in order to eliminate impurities after that this

solution was dialyzed in a cellulose membrane tube (MWCO 4501) at room temperature for 4 d against distilled water changed daily to remove the salt residue and centrifuged at 10,000 rpm for 10 min. The obtained SF solution was lyophilized at -40 °C and then freeze-dried by using a freeze-drier machine to obtain the regenerated SF sponges.

6 wt.-% of the regenerated SF sponge was dissolved in distilled water under mechanical stirring for 2 h and then filtrated through filtrated paper to separate the non-dissolved SF sponge. The concentration of the SF solution was estimated by weighing 1 ml of SF solution that was dried. The SF solution was adjusted by distilled water to obtain the concentration of SF solution, estimated as 5 wt.-%. Silver nitrate ($AgNO_3$) at various contents (0.5, 1.0, 1.5, 2.0 and 2.5 % by weight of the dry silk fibroin powder) was added into the previous solution and continuously stirred at various times to allow the formation of silver nanoparticles (nAgs) within these solutions.

3.3.1 Preparation of the Hydrogels

3.3.2.1 Preparation of nAg-Loaded Gelatin Hydrogel Pads

Glutaraldehyde aqueous solutions (GTA) at various concentrations (0.5, 1, 3, 5, 7 and 9 µl/ml of gelatin solution) were mixed with an $AgNO_3$-containing gelatin solution that had been aged for a proper time interval under mechanical stirring for 30 min. Each of the resulting solutions (6 ml) was then cast on a polytetrafluoroethylene (PTFE) mold (square projection with 5.5 cm × 5.5 cm and 0.5 cm in depth), followed by air-drying at room temperature for 24 h to allow the solidification. After that, the gelatin hydrogel pads were kept in an oven at 120 °C for 4 d in order to complete the cross-linking reaction and to remove as much the residual solvent from the gelatin hydrogels as possible. The cross-linked gelatin hydrogels were immersed in 0.4 %w/v sodium metabisulfite ($Na_2S_2O_5$) aqueous solution for 24 h, washed four times in distilled water and then air-dried for about 24 h. The thickness of the hydrogel pads in their dry state was about 150-170 µm.

3.3.2.2 Preparation of nAg-Loaded Silk Fibroin Films

Each of the nAg containing SF solutions (30 ml) that had been loaded with different $AgNO_3$ contents was casted onto a polystyrene petri dish (85 mm in diameter) and followed by air drying at room temperature for 3 d. The nAg-loaded SF films were treated with 90 vol.-% of methanol aqueous solution for 10 min to induce SF β-sheet induction and insolubility. After the treatment step, these films were dried at room temperature for 1 h. The thickness of the films in their dry state was about 200-240 μm.

3.3.3 Characterization

3.3.3.1 Formation of nAg

The formation of nAg in the $AgNO_3$-containing gelatin and silk fibroin solutions that had been aged for various time intervals was confirmed by monitoring the appearance of the surface plasmon band using a Shimadzu UV-2550 UV-visible spectrophotometer (UV-vis). The size of the as-formed nAg along with its distribution was characterized by a JEOL JEM-2100 transmission electron microscope (TEM).

3.3.3.2 Water Retention and Weight-Loss Behavior

The nAg-loaded gelatin hydrogel pads in their dry state were cut into circular discs (15 mm in diameter) and weighed in order to ascertain the initial dry weight (w_i). After that, the specimens were immersed in 50 ml of each of the three media [i.e., acetate buffer solution (pH 5.5) at 32 °C, distilled water (pH 6.5) at 37 °C, simulated body fluid (SBF) buffer and phosphate buffer saline solutions (pH 7.4) at 37 °C] for various time intervals. After the submersion, each specimen was blotted with a piece of tissue paper to remove the excess amount of the medium on its surface and then immediately weighed to determine the weight of the specimen in its wet, swollen state (w_s). The specimens were then dried in an oven until of a constant weight to obtain the dried weight (w_d). The percentages of water retention and weight loss were calculated as follows:

$$\text{Water retention (\%)} = \frac{w_s - w_d}{w_d} \times 100, \qquad (1)$$

and weight loss (%) = $\frac{w_i - w_d}{w_d} \times 100$ (2)

3.3.3.3 Number-Average Molecular Weight of Chain Segments Between Cross-linking Points and Cross-link Density

The cross-link densities of the nAg-loaded gelatin hydrogel pads were determined based on the data obtained from the water retention behavior of the specimens in distilled water. To calculate for the cross-link density values, the number-average molecular weight of the chain segments between cross-linking points, M_c, was the first to be determined from the equilibrium water retention values which had been performed in distilled water at 37 °C, according to the Flory-Renher equation:

$$M_c = -\rho_G V_1 \upsilon_G^{1/3} / [(\chi \upsilon_G^2 / 2) + \ln(1 - \upsilon_G) + \upsilon_G], \quad (3)$$

where ρ_G is the density of gelatin (i.e., 1.35 g·cm^{-3}), V_1 is the molar volume of water, χ is the Flory-Huggins interaction parameter between water and gelatin (i.e., 0.49 ± 0.05 [26]) and ν_G is the volume fraction of gelatin in hydrogel specimens in their equilibrium swollen state, which could be calculated from the following equation:

$$\upsilon_G = W_0 \rho_W / [W \rho_G - W_0 (\rho_G - \rho_W)], \quad (4)$$

where W_0 is the initial dry weight of the specimens, ρ_w is the density of water and W is the weight of the specimens in their equilibrium swollen state (after the removal of the excess water on the surface of the specimens by tissue papers). The cross-link density in terms of the number of elastically effective chains per unit volume of the gelatin hydrogel network, V_e, could then be expressed as an inverse function of M_c as follows:

$$V_e = \rho_G N_A / M_c, \quad (5)$$

where N_A is the Avogadro's number.

3.3.3.4 In Vitro Degradation Test

Protease XIV enzyme (from *Streptomyces griseus*, cat#P5147, Sigma, MO) was used in this experiment to study enzymatic degradation of SF films. Enzyme was dissolved in simulated body fluid buffer solution (SBF, pH 7.4) which has ion concentrations nearly equal to those of human blood plasma to prepare the enzyme solution (1.0 mg/ml). And the nAg-loaded SF films that had been loaded

with 2.5 wt.-% $AgNO_3$ in their dry state were cut into circular discs (15 mm in diameter) and weighed in order to ascertain the initial dry weight (w_i). After, the samples were immersed in 2 ml of both SBF and the enzyme solution (SBF with enzyme) and then incubated at 37 °C for various time intervals (1, 2, 3, 4, 5, 6 and 7 d). The enzyme solutions were changed daily because protease might be less effective after 1 d. At each time, the samples were taken out from media and were then dried in an oven until of a constant weight to obtain the final weight (dry) after specified days of incubation (w_d). The percentage of remaining weight of the nAg-loaded SF films was calculated as follows:

$$\text{Remaining weight (\%)} = \frac{W_d}{W_i} \times 100 \qquad (6)$$

3.3.3.5 nAg Release Assay

First, the actual amount of nAg in the gelatin and silk fibroin hydrogels was determined. Specifically, the uncross-linked nAg-loaded gelatin and silk fibroin pads in their dry state (cut into circular disc specimens of 15 mm in diameter) were dissolved in 50 ml of each respective medium under mechanical stirring. The actual amount of nAg within the hydrogels was then quantified by a Varian SpectrAA-300 atomic absorption spectroscope (AAS). In the release assay, disc specimens of the nAg-loaded gelatin and silk fibroin hydrogel pads in their dry state were placed in 50 ml of each of media [i.e., acetate buffer solution (pH 5.5) at 32 °C, distilled water (pH 6.5) at 37 °C, simulated body fluid (SBF) buffer and phosphate buffer saline solutions (pH 7.4) at 37 °C] for various time intervals (i.e., 1, 3, 6, 12, 24, 72, 120, 168 and 216 h). At each time point, the medium was totally removed and an equal amount of the fresh medium was replaced. The amount of the released nAg in the withdrawn medium (i.e., sample solution) was determined by AAS.

3.3.3.6 Antimicrobial Activity Assay

3.3.3.6.1 Microorganisms

Six bacterial strains used were *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923, *Methicillin-resistant*

Staphylococcus aureus DMST 20654, *Staphylococcus epidermidis* ATCC 12228, *Streptococcus agalactiae* DMST 17129, *Streptococcus pyogenes* and one fungal strain, *Candida albicans* ATCC 10231. All of these were human pathogenic microorganisms and kindly supported by National Institute of Health, Department of Medical Sciences, Ministry of Public Health, Thailand and Department of Biotechnology, Faculty of Science, Ramkhamhaeng University.

3.3.3.6.2 Agar Dilution Method

The minimal inhibition concentration (MIC) values of $AgNO_3$ were determined by an agar dilution method against the microorganism strains that were usually the causative agents on the skin wound. The two-fold serial dilutions of $AgNO_3$ were mixed with the sterilized Muller-Hinton broth and agar to the final volume of 5 ml with the final concentration from 10 mg/ml to 0.6 μg/ml and immediately poured into petri dishes (50 mm in diameter). Ten microliters of 10^4 CFU/ml of each strain were spotted and inoculated onto the plates. The plates were then incubated at 37 °C for 24 h. The inhibition of the microbial growth was compared with the growth in the control plate. After 24 h of incubation, the MIC was defined as the lowest concentration of $AgNO_3$ inhibiting the visible growth of each organism on the agar plate and the MIC measurement was done in triplicate to confirm the value of MIC for each strain.

3.3.3.6.3 Effects of The nAg Containing SF Solutions on Microbial Growth

The antimicrobial activity of the nAg containing SF solutions at various $AgNO_3$ concentrations was investigated. Each of activated microbial strains in Luria-Bertani broth at 10^8 CFU/ml concentration was seeded in a 96-well tissue-culture polystyrene plate (TCPS; Biokom Systems, Poland) and then inoculated at 37 °C under shaking until the optical density (O.D.) at 600 nm equal to 0.1. Each of the sterilized sample solutions (30 μl) was added into wells containing microbial culture in LB broth. For the control, 30 μl of the sterilized LB broth was added instead of the sterilized sample solutions. The tested solutions containing microorganisms were immediately measured the optical density at 600 nm and incubated 37 °C under shaking. These solutions were taken to measure the optical

density at 600 nm every hour (1-8 h). The optical density was calculated to the percentage of relative viability of microorganisms as followed:

$$\%\text{Relative viability of microorganisms} = \frac{\text{O.D.}_{\text{sample}}}{\text{O.D.}_{\text{control}}} \times 100 \quad (7)$$

3.3.3.6.4 The Colony Count Method

The bacteria from cultures grown in tryptic soy broth (TSB) at 37 °C for 24 h were added into 4.5 ml of TSB and the optical densities (ODs) of the bacterial suspensions were measured at the wavelength of 600 nm using a Thermo Scientific GENESYS 20 4001/4 spectrophotometer. At the bacterial concentration of 10^8 cells·ml^{-1}, the ODs of the bacterial suspensions for *S. aureus* and *P. aeruginosa* were 0.2, while that for *E. coli* was 0.4. These bacteria were serially diluted with a NaCl aqueous solution to obtain bacterial suspensions with bacterial concentrations in the range of 10^8-10^5 cells·ml^{-1}. Prior to the assessment, both the neat and the nAg-loaded gelatin hydrogel pads were sterilized in 70 vol.-% ethanol aqueous solution for 30 min, air-dried at room temperature (25 ± 2 °C) in a sterilized hood and kept in sterilized bags. After air-drying, 1 ml of each of the dilute bacterial suspensions was put into a bag containing a sterilized hydrogel specimen (weighing around 0.4 g) and incubated at 37 °C for 24 h. The bacteria were then washed out from the specimen by immersing the specimen in 100 ml of distilled water in a flask that was shaken by a Burrell Scientific Model AA Wrist-Action® shaker, operating at 400 rpm, for 5 min. Exactly 0.1 ml of the washing solution was withdrawn and then plated onto Difco™ Mueller-Hinton agar in a Petri dish using an Advanced Instruments Spiral Biotech Autoplate 4000 Spiral Plater. The plates were then incubated at 37 °C for 24 h. Finally, the numbers of bacterial colonies were counted and the obtained data were used to calculate the reduction in the number of bacterial colonies (i.e., the bacterial growth inhibition), according to the following equation:

$$\text{Bacterial growth inhibition (\%)} = (B - A)/B \times 100, \quad (8)$$

where *A* and *B* are the numbers of bacterial colonies (i.e., colony forming unit per ml; CFU·ml^{-1}) for the plates that had been smeared with washing solutions from the nAg-loaded and the neat gelatin hydrogel specimens, respectively.

3.3.3.6.5 Agar Disc Diffusion Method

The US Clinical and Laboratory Standards Institute (CLSI) disk diffusion method was used to assess the antimicrobial activity of the nAg-loaded SF films. The neat SF film was used as the control. The 18 h microbial culture in Muller-Hinton broth was diluted to approximately 10^5 CFU/ml with 0.85 %w/v normal saline solution. The microbial solutions were spread over the Muller-Hinton agar plates. The tested films were cut into circular discs with 15 mm diameter and sterilized with 70 % ethanol aqueous solution for 30 min. Furthermore, all samples were placed on microorganisms-cultured agar plates and incubated at 37 °C for 24 h. The inhibition zone was monitored and the mean value was calculated.

3.3.3.7 The Cell Study of Hydrogels

3.3.3.7.1 Indirect Cytotoxicity Evaluation

The indirect cytotoxicity evaluation of the nAg-loaded gelatin hydrogel pads was conducted in adaptation from the ISO10993-5 standard test method, using normal skin fibroblasts as reference cells. The cells were first cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, USA), supplemented with 10 % fetal bovine serum (FBS; Biochrom AG, Germany), 1 % L-glutamine (Invitrogen Corp., USA) and 1% antibiotic and antimycotic formulation [containing penicillin G sodium, streptomycin sulfate, and amphotericin B (Invitrogen Corp., USA)]. The culture medium was changed every 3 d and the cultures were maintained at 37 °C in a humidified atmosphere containing 5 % CO_2.

The hydrogels in their dry state were first cut into circular discs (15 mm in diameter), weighing about 0.02 g each. Before testing, the specimens were each sterilized by 70 vol.-% ethanol for 30 min. Extraction media were then prepared by immersing the specimens individually in serum-free medium (SFM; containing DMEM, 1% L-glutamine, 1 % lactalbumin, and 1% antibiotic and antimycotic formulation) at the extraction ratio of 10 mg·ml^{-1} in wells of a 96-well tissue-culture polystyrene plate (TCPS; Biokom Systems, Poland) for 1, 3 and 7 d,

respectively. The normal skin fibroblasts from the cultures were trypsinized [0.25 % trypsin containing 1 mM EDTA (Invitrogen Corp., USA)], counted by a hemocytometer (Hausser Scientific, USA), and seeded at a density of about 10,000 cells/well on TCPS in serum-containing DMEM for 16 h to allow cell attachment. The cells were then starved with SFM for 24 h. After that, the medium was replaced with an extraction medium, and the cells were re-incubated for 24 h. Finally, the viability of the cells cultured by each of the as-prepared extraction media was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The viability of the cells cultured by fresh SFM was used as control.

The MTT assay is based on the reduction of yellow tetrazolium salt to purple formazan crystals by dehydrogenase enzymes secreted from the mitochondria of metabolically active cells. The amount of the as-formed purple formazan crystals is related to the number of viable cells in a linear manner. First, each culture medium was aspirated and replaced with 25 μl/well of MTT solution at 5 $mg \cdot ml^{-1}$ for a 96-well TCPS. Secondly, the plate was incubated for 3 h at 37 °C. The solution was then aspirated, and 100 μl/well of DMSO was added to dissolve the formazan crystals. Finally, after 3 min of rotary agitation, the absorbance at a wavelength of 550 nm representing the viability of the cells was measured by a SpectraMax M2 Microplate Reader.

3.3.3.7.2 Morphology of Seeded and Cultured Cells

The nAg-loaded gelatin hydrogel pads were cut into circular disc specimens (about 15 mm in diameter and about 0.02 g each). All specimens were sterilized in 70 vol.-% ethanol aqueous solution for 30 min. Normal skin fibroblasts from the culture (10^{th} to 15^{th} passages) were seeded or cultured on the surfaces of the disc specimens at a density of 1.5×10^4 cells/well in wells of a 24-well TCPS at 37 °C under a humidified atmosphere containing 5 % CO_2 for 5 h, 1 d and 5 d, respectively. The surfaces of disc specimens from the neat gelatin hydrogel pads were used as internal control and those of glass slides were used as external control. The 5 h seeding period represented the attachment stages of the cells and the 1 and 5 d culturing periods represented the proliferation stage of the cells. Following each seeding or culturing period, morphology of the cells was observed by a JEOL JEM-5600LV scanning electron microscope (SEM). After removal of the culture

medium, the cells on the specimen surfaces were fixed with 400 μl/well of 3 vol.-% glutaraldehyde aqueous solution which had been diluted from the as-received GTA with PBS at room temperature (25 ± 2 °C) for 30 min. The cell-covered specimens were then washed twice in distilled water. Subsequently, they were dehydrated by submersion in graded ethanol aqueous solutions and pure ethanol (at 400 μl/well) for 2 min at each concentration and then dried again in 100 % hexamethyldisilazane (HMDS; Sigma-Aldrich, USA) for 5 min. The completely-dried specimens were mounted on copper stubs and sputter-coated with gold for 4 min prior to the SEM observation.

3.3.3.8 Mechanical Property Evaluation

The nAg-loaded gelatin hydrogel pads in their dry state were cut into rectangular shapes (5 mm in width × 10 mm in length). The test specimens were first immersed in acetate buffer solution at 37 °C for 1 d. After the submersion, the specimens were blotted with a piece of tissue paper and then characterized by a Lloyd LRX universal testing machine. The load cell, the crosshead speed and the gauge length of the specimens were 500 N, 10 mm/min and 30 mm, respectively.