CHAPTER IV
DEVELOPMENT OF GELATIN HYDROGEL PADS AS ANTIBACTERIAL WOUND DRESSINGS

4.1 Abstract

Gelatin hydrogel pads were prepared from 10 wt.-% gelatin solution containing 2.5 wt.-% $AgNO_3$ in 70 vol.-% acetic acid by a solvent-casting technique. The $AgNO_3$-containing gelatin solution was aged under a mechanical stirring for various time intervals to allow for the formation of silver nanoparticles (nAgs). The formation of nAgs was monitored by a UV-Vis spectrophotometer. The morphology and size of nAgs were characterized by transmission electron microscopy (TEM). To improve the water resistance of the hydrogels, various contents of glutaraldehyde (GTA) were added to the $AgNO_3$-containing gelatin solution to cross-link the obtained gelatin hydrogels. These hydrogels were tested for their water retention and weight loss behavior, release characteristic of the as-loaded silver and antibacterial activity against Gram-negative *Escherichia coli* and Gram-positive *Staphylococcus aureus*. The $AgNO_3$-containing gelatin solution that had been aged for 5 d showed the greatest number of nAgs formed. The size of these particles, based on the TEM result, was 10-11 nm. With an increase in the GTA content used to cross-link the hydrogels, the water retention, the weight loss and the cumulative amount of silver released were found to decrease. Finally, all of the nAg-loaded gelatin hydrogels could inhibit the growth of the tested pathogens, which confirmed their applicability as antibacterial wound dressings.

(**Key-words**: Gelatin hydrogels; Silver nanoparticles; Antibacterial activity)

4.2 Introduction

The major problems of burn wound management and therapy are bacterial infection and wound sepsis, which may, in the worst cases, lead to mortality in patients. One approach for mitigating these problems is the use of antibacterial

agents (Burkatovskaya *et al.*, 2006). The most active ones are silver-based materials. Moyer *et al.* (1965) reported that a 0.5 % solution of silver nitrate ($AgNO_3$) was the lowest concentration that remained active against bacteria *in vitro*, and appeared to have no toxic effect on growing epidermal cells *in vivo*. Silver sulfadiazine (i.e., silver[(4-aminophenyl)sulfonyl](pyrimidin-2-yl)azanide, $C_{10}H_9AgN_4O_2S$) has been considered as the gold standard in the treatment of burn wounds (Cho Lee *et al.*, 2005). The antibacterial activity of silver-based materials is generally attributed to four mechanisms. Ionic silver binds to the bacterial cell membrane (damaging it and/or interfering with various receptors); interferes with bacterial electron transport (impeding the production of adenosine triphosphate, the cell's energy "currency"); binds to bacterial DNA (impairing cell replication); and causes the intracellular formation of insoluble compounds with certain nucleotides, proteins and/or the amino acid histidine (making them unavailable as intracellular "building blocks") (Klasen, 2000; Hermans, 2006; Atiyeh *et al.*, 2007,).

Recent reports, however, have indicated that many silver-based compounds, i.e., $AgNO_3$ and silver sulfadiazine, produce toxicity or other adverse effects. These drawbacks are usually caused not by the silver ions but by the associated anions such as the nitrate and the sulfadiazine. $AgNO_3$ is hypotonic and may cause serious hyponatremia and hypochloremia (Poon and Burd, 2004; Vlachou *et al.*, 2007). Moreover, both silver compounds cause a gray and black discoloration (Klasen, 2000). To prevent these problems, much attention has been given to pure atomic or ionic silver. Silver nanoparticles (nAg) have remarkably unusual physical, chemical and biological properties, due to their large surface area to volume ratio (Lopez *et al.*, 2005). Their small size also provides greater particulate mobility and transdermal penetration (Suzuki *et al.*, 2001; Shahverdi *et al.*, 2007).

Hydrogels are networks of polymer chains that are water-insoluble. Such internal network structures may result from physical or chemical domains that remain intact, despite being surrounded by water molecules. A number of synthetic or natural polymeric materials can be fabricated into hydrogels, rendering them diverse properties. Applications of hydrogels in medicine are, for example, as carriers for sustained release of drugs or other therapeutic substances and matrices for repairing and/or regenerating defected or lost tissues (Young *et al.*, 2005; Serena

and Chakravarthy, 2007). Moreover, the use of hydrogels as either passive or active wound dressings has increased dramatically because they offer moist conditions which facilitate wound healing. Active wound dressings, which can deliver drugs or other therapeutic substances to the wound site, are becoming very popular (Draye *et al.*, 1998; Choi *et al.*, 1999; Ulubayram, 2001). Much attention has also been paid to the development of the type of wound dressings with antibacterial activity.

Among the various natural polymeric materials, gelatin is available in abundance and, therefore, is comparatively cheap. It is a biopolymer that is readily obtained by a controlled hydrolysis of collagens, which are the most abundant structural fibrous insoluble proteins found in skin, tendons, cartilages, bones and connective tissues. Its composition and biological properties are almost identical to its precursors. Gelatin has been widely used in pharmaceutical and medical fields as sealants for vascular prostheses, plasma expanders, ingredients in drug formulations, carriers for delivery of drugs or other therapeutic substances (Einerson *et al.*, 2003; Konishi, 2003; Konishi, 2005) and, especially, as wound-dressing materials (Tabata, 1994; Tabata *et al.*, 1998; Lien *et al.*, 2008). However, gelatin, in general, exhibits a poor mechanical property that is too brittle when fully dried or too soft when fully wet and is easily soluble in aqueous media. To mitigate this, cross-linking can be introduced (Draye *et al.*, 1998; Mwangi *et al.*, 2004; Lien *et al.*, 2008).

The preparation of nAg-containing gelatin hydrogel pads to be used as antibacterial wound dressings is, therefore, the purpose of this study. The gelatin hydrogels were chemically cross-linked with glutaraldehyde to improve their mechanical integrity in the wet state. The gelatin hydrogels containing nAg were then characterized for their antibacterial activity against two commonly studied pathogens – Gram-positive *Staphylococcus aureus* and Gram-negative *Escherichia coli* – in order to elucidate their potential for use as antibacterial wound dressings. Cytotoxicity of the obtained gelatin hydrogels was evaluated *in vitro* by incubating samples for prolonged periods in the presence of suitable target cells.

4.3 Experimental

4.3.1 Materials

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). All other chemicals were of analytical reagent grade and used without further purification.

4.3.2 Sample Preparation

4.3.2.1 Preparation of nAg-Containing Gelatin Solution

$AgNO_3$ at 2.5 % by weight of the dry gelatin powder was dissolved in 70 vol.-% acetic acid aqueous solution. 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.

4.3.2.2 Preparation of nAg-Loaded Gelatin Hydrogel Pads

Glutaraldehyde aqueous solutions 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 (Vandervoort, 2004) 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.

4.3.3 Characterization

4.3.3.1 Formation of nAg

The formation of nAg in the $AgNO_3$-containing gelatin solution 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).

4.3.3.2 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 or simulated body fluid (SBF) buffer solution (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:

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

and

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

4.3.3.3 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 (Apostolov *et al.*, 2000):

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

where ρ_G is the density of gelatin (i.e., 1.35 g·cm^{-3} (Bertoldo *et al.*, 2007)), 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 (Apostolov *et al.*, 2000)) and υ_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 (Bajpai and Sharma, 2006) 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.

4.3.3.4 Preparation of Media

- *Acetate Buffer*

Acetate buffer was chosen to simulate human skin pH condition of 5.5. To prepare 1000 ml of the acetate buffer solution, 150 g of sodium acetate was dissolved in ~250 ml of distilled water. Exactly 15 ml of glacial acetic acid was then added very slowly into the sodium acetate aqueous solution. Finally, distilled water was added into the solution to fill the volume.

- *Simulated Body Fluid Buffer*

Simulated body fluid buffer has ion concentrations nearly equal to those of human blood plasma and is buffered at pH 7.40. To prepare 1000 ml of the simulated body fluid buffer, 7.996 g of sodium chloride (NaCl) was dissolved in~500 ml of distilled water. Reagents (i.e 0.350 g of sodium hydrogen carbonate, 0.224 g of potassium chloride, 0.228 g of dipotassium hydrogen phosphate, 0.305 g of magnesium chloride, 40 ml of hydrochloric acid, 0.278 g of

calcium chloride, 0.071 g of sodium sulfate, 6.057 g of tris (hydroxymethyl)aminomethane were added into previous solution one by one after the former reagent was completely dissolved. The temperature of the obtained solution was adjusted at 36.5 °C with a water bath, and pH of this solution was adjusted at pH 7.40 by stirring the solution and titrating 1N-HCl solution. Finally, the total volume of the solution to 1000 ml was adjusted by adding distilled water.

- *Phosphate Buffer Saline*

To prepare 1000 ml of stock of phosphate buffer saline, the reagents (i.e 8 g of sodium chloride, 0.2 g potassium chloride, 1.44 g of disodium hydrogen phosphate and 0.24 g potassium dihydrogen phosphate) were dissolved in 800 ml of distilled water. The pH of this solution was adjusted to 7.4 using hydrochloric acid and added distilled water to a final volume of 1000 ml.

4.3.3.5 nAg Release Assay

First, the actual amount of nAg in the gelatin hydrogels was determined. Specifically, the uncross-linked nAg-loaded gelatin 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 hydrogel pads in their dry state were placed in 50 ml of each respective medium for various time intervals (i.e., 1, 3, 6, 12, 24, 72, 120 and 168 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.

4.3.3.6 Antibacterial Evaluation

The US Clinical and Laboratory Standards Institute (CLSI) disc diffusion method was used to assess the antibacterial activity of the nAg-loaded gelatin hydrogel pads. The neat hydrogels were used as the control group. Their antibacterial activity was tested against Gram-positive *Staphylococcus aureus* (*S. aureus*, ATCC 25023) and Gram-negative *Escherichia coli* (*E. coli*, ATCC 25922). Vancomycin and gentamicin were used as antibacterial drugs for *S. aureus* and *E.*

coli, respectively. The specimens (circular discs of 15 mm in diameter) and the drugs were placed on Difco™ Mueller-Hinton agar in a Petri dish and then incubated at 37 °C for 18 h. Finally, the agar plate was photographed to evaluate the antibacterial activity of each specimen. If inhibitory concentrations were reached, there would be no growth of the microbes, which could be seen as clear zones around the disc specimens.

4.3.3.7 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 nAg-loaded gelatin 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 $mg \cdot mL^{-1}$ 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 1mM 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.

4.3.3.8 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.

4.3.3.9 Statistical Analysis

Data were presented as means ± standard errors of means (n = 3). A one-way ANOVA was used to compare the means of different data sets, and equal variances assumed using Scheffé's method. The statistical significance was accepted at a 0.05 confidence level.

4.4 Results and Discussion

4.4.1 Formation of nAg in $AgNO_3$-Containing Gelatin Solution

In the present study, the gelatin solution in 70 vol.-% acetic acid solution that contained $AgNO_3$ was prepared. Silver nanoparticles (nAgs) were subsequently formed in the $AgNO_3$-containing gelatin solution after it had been aged for various time intervals. The reduction of Ag^+ into its metallic form has been shown to be accomplished by a number of methods, i.e., chemical reduction,

irradiation with γ-ray and ultrasonication (Wang and Asher, 2001; Zhang *et al.*, 2001; Lu *et al.*, 2003). The formation of nAg in the $AgNO_3$-containing gelatin solution after it had been aged for various time intervals can be identified simply by a change in the color of the solution and by a change in the UV-visible absorption spectrum (see later). Such changes were not observed in the $AgNO_3$/acetic acid solution, no matter how long the solution had been aged.

Figure 1 shows the appearance of the neat gelatin solution and the $AgNO_3$-containing gelatin solution that had been aged for various time intervals. Evidently, the color of the neat gelatin solution was light yellow. The presence of $AgNO_3$ in the acetic acid solution prior to the dissolution of gelatin powder caused the resulting $AgNO_3$-containing gelatin solution to become slightly darker yellow (result not shown). After the $AgNO_3$-containing gelatin solution had been aged for 1 d, the color of the solution changed to light brown. Evidently, the color of the solution became darker and darker with further increase in the aging period to finally become dark brown after it had been aged for 5 d. Though not shown, the color of the neat gelatin solution did not change no matter how long the solution had been aged.

Figure 2 shows a spectrophotometrical evidence for the formation of nAg. It has been shown in the literature that the formation of nAg can be observed from the appearance of the surface plasmon resonance absorption peak that centers around 416-445 nm in the UV spectrum, with the peak position hypothetically decreasing with a decrease in the size of the as-formed nAg (Arai *et al.*, 2001; Yang *et al.*, 2003; Frattini *et al.*, 2005; Lee *et al.*, 2005). Such a peak was not observed in the UV spectrum of the $AgNO_3$-containing gelatin solution that had been aged for only 1 d. Increasing the aging period to 2 d resulted in a noticeable, but rather vague, change to the baseline of the spectrum. The surface plasmon peak centering around 430-435 nm was clearly observed for the $AgNO_3$-containing gelatin solution that had been aged for at least 4 d. Evidently, both the absorbance and the position of the surface plasmon peak increased with an increase in the aging period, the results that respectively indicated the increase in both the number and the size of the as-formed nAg with an increase in the aging period (Yang *et al.*, 2003; Jin *et al.*, 2005; Lee *et al.*, 2005; Son *et al.*, 2006)

Figure 3 shows representative TEM images of the as-formed nAg in the $AgNO_3$-containing gelatin solution that had been aged for various time intervals ranging from 4 to 7 d. Both the number and the size of these particles were analyzed by constructing histograms of the particulate diameters against the frequency, as shown along side each respective TEM image. For the solution that had been aged for 4 or 5 d, the size of the as-formed nAg was 10 or 11 nm on average. At these conditions, the size distribution was quite narrow. For the solution that had been aged for 6 or 7 d on the other hand, nAg with much larger diameters (i.e., 20 or 28 nm on average) and wider size distribution were apparent. Aggregation of the growing particles that were in close proximity to one another was postulated as the main reason for such observations.

Based on the results shown in Figures 1 to 3, it is obvious that the presence of gelatin in the $AgNO_3$/acetic acid solution was the main factor responsible for the formation of nAg. Even though about half of the amino acid residues in gelatin molecules contain relatively hydrophobic side groups (i.e., glycine, alanine, proline and hydroxyproline, which all accounted for about 54% based on the dry weight of the protein (Songchotikunpan *et al.*, 2008)), some amino acid residues, such as glutamic acid, aspartic acid and arginine (which all accounted for about 24 % based on the dry weight of the protein (Songchotikunpan *et al.*, 2008)), contain either carboxylic acid or neutral amino groups that can interact with the Ag^+ cations directly, which may lead to the complexation of Ag^+ to such groups (Zhang *et al.*, 2001; Lu *et al.*, 2003). In subsequent studies, the $AgNO_3$-containing gelatin solution that had been aged for 5 d was chosen for further fabrication into films by solvent-casting technique.

4.4.2 Characterization of nAg-Loaded Gelatin Hydrogel Pads

4.4.2.1 Water Retention and Weight Loss Evaluation

Without cross-linking, a solvent-cast gelatin film can dissolve easily in an aqueous medium. Here, glutaraldehyde (GTA) was used as a cross-linking agent for making nAg-loaded gelatin hydrogel pads, simply because of its known efficiency as a cross-linking agent for gelatin (Zhang *et al.*, 2006; Songchotikunpan *et al.*, 2008) and, particularly, its inexpensiveness. The

measurements for the water retention and the weight loss were used to assess the extent of the cross-linking of the gelatin hydrogels that had been treated with various contents of GTA. Three types of the aqueous medium was used for these purposes: they are acetate buffer solution (pH 5.5), distilled water (pH 6.5), and SBF buffer solution (pH 7.4). The results are graphically shown in Figures 4-6.

At a given GTA content, the water retention of the hydrogels, in any type of the medium, increased with an initial increase in the submersion time and finally became saturated with the medium after they had been submerged in the medium for about 1-3 d. At the same time, it was observed that the amount of the retained water, at any given time point, decreased with an increase in the GTA content used to cross-link the hydrogels. For the hydrogels that had been submerged in the acetate buffer for 7 d, the amounts of the retained water were found to decrease from about 866 % at 0.5 μl/ml of GTA to saturate at about 251 % at 7 and 9 μl/ml of GTA. In distilled water, such values decreased from about 1137 % at 0.5 μl/ml of GTA to about 257 % at 9 μl/ml of GTA. In the SBF buffer, the values decreased from about 997 % at 0.5 μl/ml of GTA to about 237 % at 9 μl/ml of GTA. Evidently, the amount of the water retained in the hydrogels after they had been submerged in distilled water was the greatest.

On the other hand, the loss in the weight of the hydrogels, at any given GTA content and in any type of the medium, increased with an initial increase in the submersion time and, in most cases, finally became constant after they had been submerged in the medium for about 1-3 d. Simultaneously, it was found that, at any given time point, the values decreased with an increase in the GTA content. Specifically, for the hydrogels that had been submerged in the acetate buffer for 7 d, the percentages of the weight loss decreased from about 40% at 0.5 μl/ml of GTA to about 9 % at 9 μl/ml of GTA. In distilled water, such values decreased from about 37 % at 0.5 μl/ml of GTA to about 11 % at 9 μl/ml of GTA. In the SBF buffer, the values decreased from about 49 % at 0.5 μl/ml of GTA to about 16 % at 9 μl/ml of GTA. Evidently, the greatest extent of the loss in the weight of the hydrogels occurred when they were submerged in SBF medium.

The decrease in both the water retention and the weight loss of the hydrogels with an increase in the GTA content was due obviously to the increase in the extent of cross-linking, as GTA molecules reacted with the free amino groups of lysine (accounted for about 3.4 % based on the dry weight of the protein (Songchotikunpan *et al.*, 2008)) and/or arginine (accounted for about 7.4 % based on the dry weight of the protein (Songchotikunpan *et al.*, 2008)) to form a network within the gelatin structure. Even though a high water retention of a hydrogel wound dressing is necessary to keep the wounds in a moist condition, which is known to promote the healing of the wounds, the GTA content used to cross-link the hydrogels could not be too low to jeopardize the physical integrity of the hydrogels (i.e., because of the weight loss could be too high) during their administration on the wounds.

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

The water retention data of the hydrogels after having been submerged in distilled water for 5 d were used to calculate the number-average molecular weight of chain segments between cross-linking points (M_c) and the cross-link density (V_e) of the cross-linked nAg-loaded gelatin hydrogel pads. The results of such calculations are summarized in Table 1. Apparently, an increase in the GTA content used to cross-link the hydrogels caused the M_c values to decrease from about 2190 g/mol at 0.5 µl/ml of GTA to about 360 g/mol at 9 µl/ml of GTA. These corresponded to a monotonous increase in the V_e values from about 3.0×10^{20} #/cm^3 at 0.5 µl/ml of GTA to about 17.7×10^{20} #/cm^3 at 9 µl/ml of GTA.

4.4.2.3 Release Characteristics of Silver

Prior to investigating the release characteristics of silver from the nAg-loaded gelatin hydrogel pads, the actual amount of silver (either in the form of the free Ag^+ cations or the as-formed nAg) in these hydrogels needed to be determined. For this purpose, the uncross-linked hydrogels that were prepared from the gelatin solution containing 2.5 wt.-% $AgNO_3$ that had been aged for 5 d were investigated for the amount of silver in each of the three media. At 2.5 wt.-% of $AgNO_3$ in the gelatin solution, the theoretical content of the as-loaded silver in the

hydrogels should be 15.49 mg/g of the hydrogels (in their dry state). Since 50 ml of each medium would be used to dissolve the hydrogels, the theoretical amount of the as-loaded silver that could be present in the medium solution would be 309.76 ppm/g of the hydrogels. Experimentally, the actual amounts of silver loaded in the hydrogels as determined by means of AAS in the acetate buffer, distilled water and the SBF buffer were 308.9 ± 1.5, 307.3 ± 2.0 and 304.5 ± 0.6 ppm/g of the hydrogels, respectively (n = 3). These accounted for 99.7 ± 0.5, 99.2 ± 0.7 and 98.3 ± 0.2 % of the initial, theoretical content of the as-loaded silver, respectively.

Figure 7 illustrates the cumulative amounts of silver, either in the form of nAg or residual free ions (Ag^+), which were released per gram of the nAg-loaded gelatin hydrogel pads that had been cross-linked with varying amounts of GTA in each of the three media as a function of the submersion time. In the acetate buffer and distilled water, the release of silver from the hydrogels could be divided into two stages: the initial rapid release within the first 24 h of submersion and the slow release for the submersion times greater than 24 h. in the SBF buffer on the other hand, the release of silver occurred more gradually over the first 7 d period, after which it occurred much more slowly. Increasing the GTA content used to cross-link the hydrogels generally resulted in the observed decrease in the cumulative amounts of silver released into the media. This correlated well with the observed decrease in both the swelling and the weight loss of the hydrogels with an increase in the GTA content. Specifically, at 24 h, the cumulative amounts of silver released in to the media were in the ranges of 228.6-266.8 ppm/g of the hydrogels in the acetate buffer, 181.3-245.8 ppm/g of the hydrogels in distilled water and 73.2-136.5 ppm/g of the hydrogels in the SBF buffer. These amounts finally increased to 281.5-305.6 ppm/g of the hydrogels in the acetate buffer, 250.9-302.0 ppm/g of the hydrogels in distilled water and 248.0-307.9 ppm/g of the hydrogels in the SBF buffer at the longest submersion time investigated (264 h).

Comparatively among the three media, the cumulative amounts of silver released from the hydrogels were greatest in the acetate buffer, followed by those in distilled water and the SBF buffer, respectively. The likely reason for such observation could be due to the difference in the protonation behavior of gelatin in these media. In the present work, type-A gelatin was used. The

isoelectric point – the pH at which a molecule carries no net electrical charge – of this type of gelatin is about 7-9. Below this point, gelatin carries a net positive charge. As a result, the repulsion between the positive charges of the protonated gelatin molecules and Ag^+ should be the main contributing factor for the greatest cumulative amounts of silver released from the hydrogels when they were submerged in the acetate buffer.

4.4.2.4 Antibacterial Activity

Here, *S. aureus* and *E. coli* were used as the model bacteria to examine the antibacterial activities of the cross-linked nAg-loaded gelatin hydrogel pads. The cross-linked gelatin hydrogel pads and the drug pellets were used as control. Table 2 shows the photographic images of the bacterium-infested agar plates which had been covered with the neat and the nAg-loaded hydrogels and the drug pellets. Table 3 summarizes the average lengths of the inhibition zones (measured from the edge of the samples to the edge of the clear zones) for all of the samples investigated. Based on the results shown in both tables, no inhibition zone against both types of bacteria was observed for the neat, cross-linked hydrogels. On the other hand, such zones were discernible for the nAg-loaded hydrogels and for the drug pellets. The inhibition zone lengths for the nAg-loaded hydrogels that had been cross-linked with 0.5 and 1 μl/ml of GTA against both types of bacteria were greatest at 2.5 mm, while they were 2 and 1.5 mm for the rest of the hydrogels against *S. aureus* and *E. coli*, respectively. These results coincided with the results on the release of silver, as the hydrogels that had been cross-linked with a low GTA content (i.e., 0.5 and 1 μl/ml) had a greater cumulative amount of the released silver than those cross-linked with a greater GTA content.

4.4.2.5 Indirect Cytotoxicity Evaluation

The potential for use of the nAg-loaded gelatin hydrogel pads as antibacterial wound dressings was further assessed with the indirect cytotoxicity assay. The effect of cross-linking on the indirect cytotoxicity of the neat gelatin hydrogels that had been cross-linked with varying amounts of GTA was first evaluated and the results are shown in Figure 8. The relative viability of normal skin fibroblasts that had been cultured with the extraction media from the neat gelatin

hydrogels was 96 %. Increasing both the GTA content used to cross-link the hydrogels and the incubation time used to prepare the extraction media from the cross-linked hydrogels resulted in a gradual and slight decrease in the relative viability of the cells. Notwithstanding, the relative viability of the cells that had been cultured with the extraction media from these cross-linked hydrogels remained relatively high at 85-93 %. The observed slight decrease in the relative viability of the cells with the increase in both of the GTA content and the incubation time could be due to the toxicity of the unreacted GTA that could have been leached out from the hydrogels (Fürst and Banerjee, 2005).

The cytotoxicity of the cross-linked nAg-loaded gelatin hydrogel pads was also evaluated using the same protocol and the results are shown in Figure 9. Without the treatment with 0.4 %w/v sodium metabisulfite aqueous solution, the relative viability of the cells that had been cultured with the extraction media from the nAg-loaded hydrogels, regardless of the GTA content used to cross-link the hydrogels, was low (i.e., 38-43 %). After the treatment with the sodium metabisulfite solution however, the relative viability of the cells increased significantly (i.e., 75-81 %), despite the slight decrease in the relative viability of the cells with the increase in both of the GTA content and the incubation time. It is hypothesized that the treatment of the nAg-loaded hydrogels with the sodium metabisulfite solution resulted in the ionic exchange between the commonly-known toxic nitrate anions (Hidalgo and Domínguez, 1998; Atiyeh *et al.*, 2007; Chen and Schluesener, 2008) with the less toxic metabisulfite anions. Such a treatment with the sodium metabisulfite solution helps restore the applicability of the nAg-loaded gelatin hydrogels as wound dressings.

4.4.2.6 Mechanical Integrity

The mechanical integrity of a wound-dressing hydrogel in its wet state is an important property determining its actual applicability. Here, the cross-linked nAg-loaded gelatin hydrogel pads that had been immersed in the acetate buffer for 1 d were tested for their mechanical properties, in terms of Young's modulus, yield strength, and elongation at yield, as function of the GTA content used to cross-link the hydrogels. The results are summarized in Table 4. While both the Young's modulus and the elongation at yield generally increased with an increase in

the GTA content, the yield strength was found to decrease. The Young's modulus was in the range of 1.5-7.4 kPa; the yield strength was in the range of 0.5-13 MPa; and the elongation at yield was in the range of 0.12-59 %. The large discrepancy in most of the property values is due to the difference in the extent of cross-linking, hence the difference in the water absorbancy, of the hydrogels.

4.5 Conclusions

In the present contribution, a gelatin solution containing silver nanoparticles (nAg) was prepared from a gelatin solution containing silver nitrate ($AgNO_3$) in acetic acid. The formation of nAg was achieved when the $AgNO_3$-containing gelatin solution was aged for at least 4 d. The formation of nAg was confirmed not only by the change in the color of the solution, but also by the observation of the surface plasmon peak in the UV spectrum at around 430-435 nm. The size of the nAg that were formed in the $AgNO_3$-containing gelatin solution that had been aged for 4-7 d, as determined by TEM, ranged between 9-28 nm. The water retention and the loss in the weight of the cross-linked nAg-loaded gelatin hydrogel pads (from the $AgNO_3$-containing gelatin solution that had been aged for 5 d) in three types of medium (i.e., acetate buffer, distilled water, and simulated body fluid buffer) were carried out to assess the effect of the glutaraldehyde (GTA) content used to cross-link the hydrogels. It was found that an increase in the GTA content resulted in the observed decrease in the values of both properties. Based on the water retention data in distilled water, the cross-link density of the hydrogels was determined and it was found to decrease with an increase in the GTA content used to cross-link the hydrogels. The total cumulative amount of silver released from the hydrogels was also found to decrease with an increase in the GTA content. The potential for use of the cross-linked nAg-loaded gelatin hydrogels as wound dressings was assessed by antibacterial activity against Gram-positive *Staphylococcus aureus* and Gram-negative *Escherichia coli* and indirect cytotoxicity against normal skin fibroblasts. The results showed that the hydrogels were effective against the two pathogens and appeared to be less toxic against the tested cells only when the hydrogels were treated with a sodium metabisulfite solution.

4.6 Acknowledgments

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Table 4.1 Number-average molecular weight of chain segments between cross-linking points (M_c) and cross-link density (V_e) of the cross-linked nAg-loaded gelatin hydrogel pads that had been immersed in distilled water at 37 °C for 5 d

Amount of glutaraldehyde (GTA) (μl/ml)	Distilled water	
	M_c (g/mol^1)	V_e (× 10^{20}) (#/cm^3)
0.5	2188 ± 185	3.0 ± 0.2
1	1301 ± 60	5.0 ± 0.2
3	567 ± 58	11.4 ± 1.1
5	372 ± 13	17.3 ± 0.6
7	368 ± 11	17.5 ± 0.6
9	364 ± 6	17.7 ± 0.1

Table 4.2 Photographic images illustrating bacterium-infested agar plates that had been covered with vancomycin or gentamicin pellets (upper panel), cross-linked gelatin hydrogel pads (left disc on lower panel) and cross-linked nAg-loaded gelatin hydrogel pads. The plates were incubated at 37 °C for 18 h

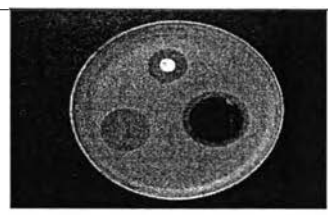
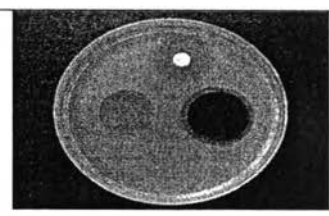
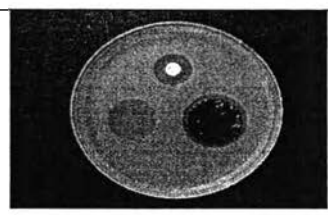
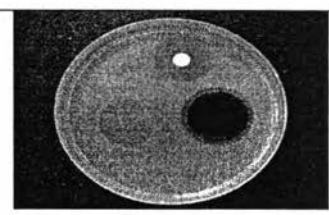
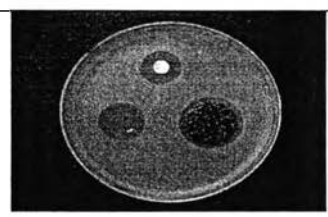
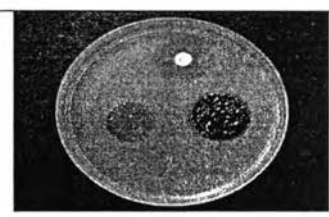
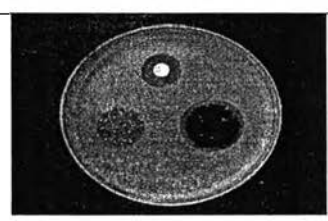
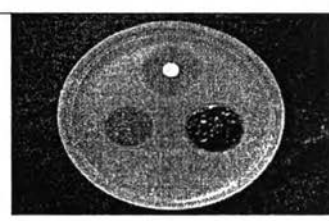
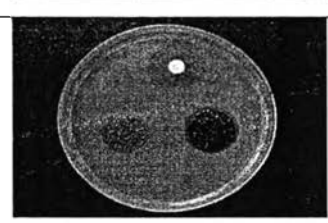
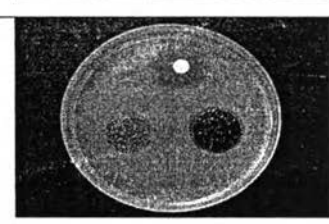
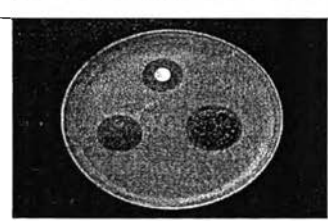
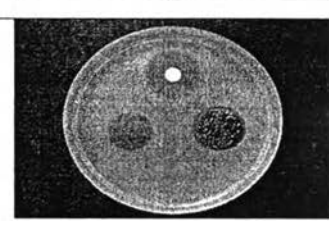
Amount of GTA (μl/ml)	Type of bacteria	
	Staphylococcus aureus	*Escherichia coli*
0.5		
1		
3		
5		
7		
9		

Table 4.3 Average lengths of the inhibition zones (measured from the edge of the samples to the edge of the clear zones observed in Table 2) for vancomycin or gentamicin pellets (A), cross-linked gelatin hydrogel pads (B) and cross-linked nAg-loaded gelatin hydrogel pads (C)

Amount of GTA (µl/ml)	Inhibition zone length (mm)					
	Staphylococcus aureus			*Escherichia coli*		
	A	B	C	A	B	C
0.5	5.5	0	2.5	5	0	2.5
1.0	5.5	0	2.5	5	0	2.5
3.0	5.5	0	2.0	5	0	1.5
5.0	5.5	0	2.0	5	0	1.5
7.0	5.5	0	2.0	5	0	1.5
9.0	5.5	0	2.0	5	0	1.5

Table 4.4 Mechanical integrity in terms of Young's modulus, yield strength and elongation at yield of cross-linked nAg-loaded gelatin hydrogel pads that had been immersed in acetate buffer at 32 °C for 1 d (n = 8)

Amount of GTA (μl/ml)	Young's modulus (kPa)	Yield strength (MPa)	Elongation at yield (%)
0.5	1.5 ± 1	13.0 ± 6	0.12 ± 0.04
1.0	2.1 ± 1	13.0 ± 5	0.13 ± 0.05
3.0	5.7 ± 2	11.0 ± 2	0.19 ± 0.24
5.0	7.1 ± 2	7.0 ± 2	0.08 ± 0.03
7.0	7.4 ± 4	9.0 ± 2	13.60 ± 3.20
9.0	6.9 ± 2	0.5 ± 0.3	58.90 ± 16.00

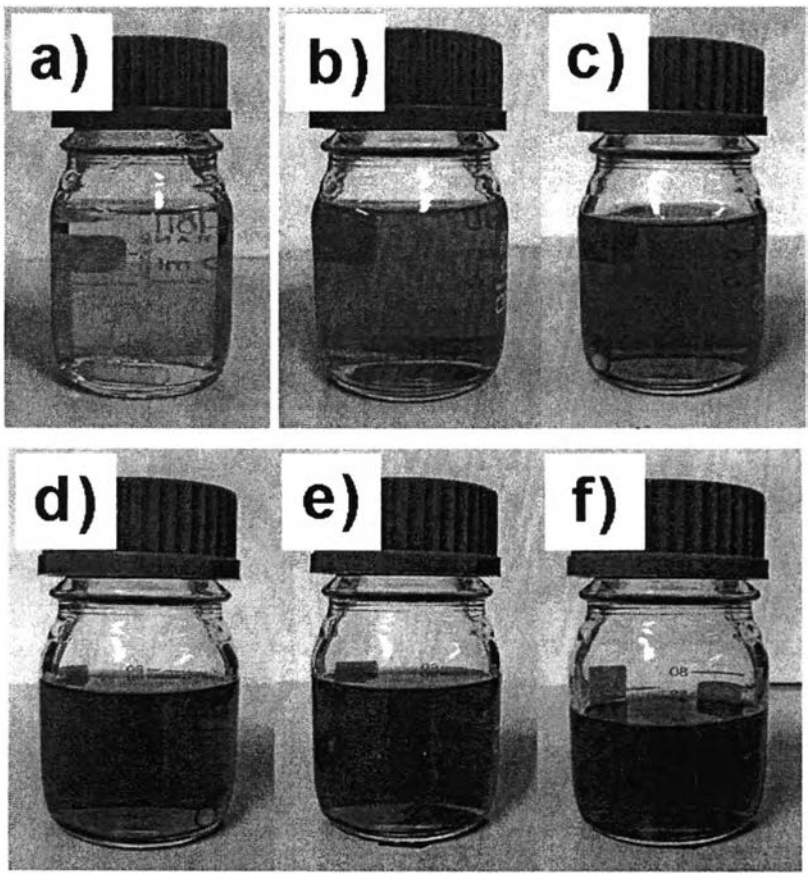


Figure 4.1 Photographic images of (a) neat gelatin solution and silver nitrate ($AgNO_3$)-containing gelatin solutions that had been aged for various time intervals: (b) 1, (c) 2, (d) 3, (e) 4 and (f) 5 d.

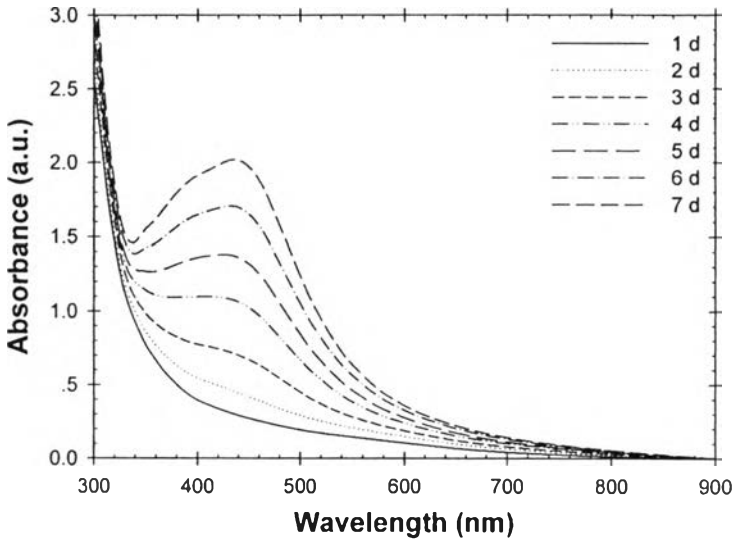


Figure 4.2 UV-visible absorption spectra of $AgNO_3$-containing gelatin solution that had been aged for various time intervals.

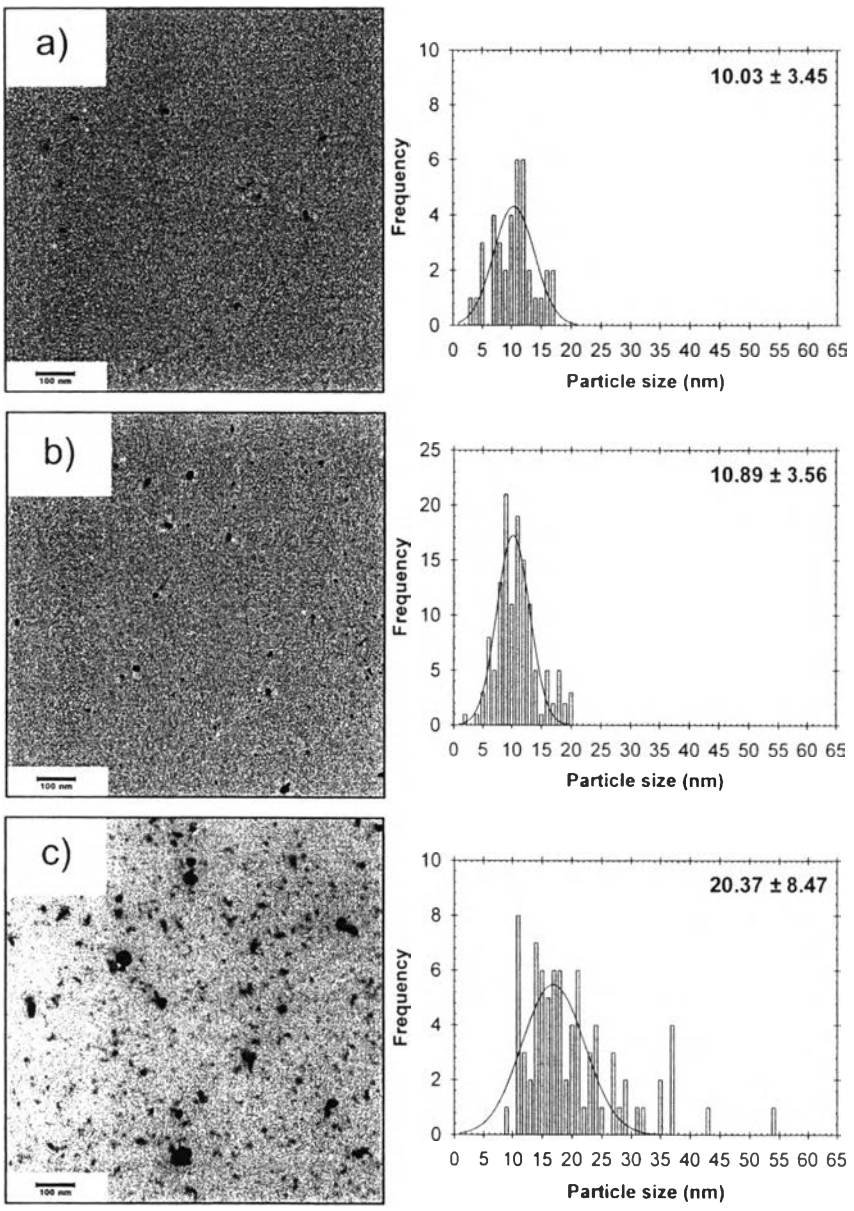


Figure 4.3 Selected TEM images (magnification = 20,000x; scale bar = 100 nm) and corresponding histograms of silver nanoparticles (nAgs) that were formed in $AgNO_3$-containing gelatin solution that had been aged for various time intervals: (a) 4, (b) 5, (c) 6 and (d) 7 d.

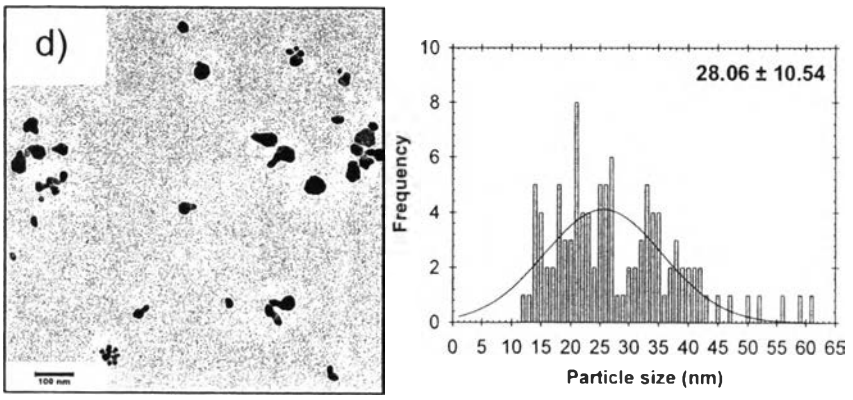
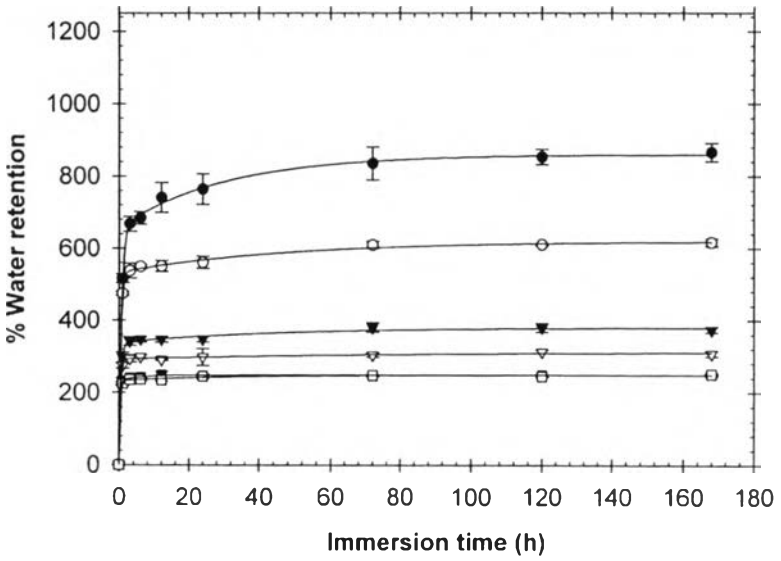
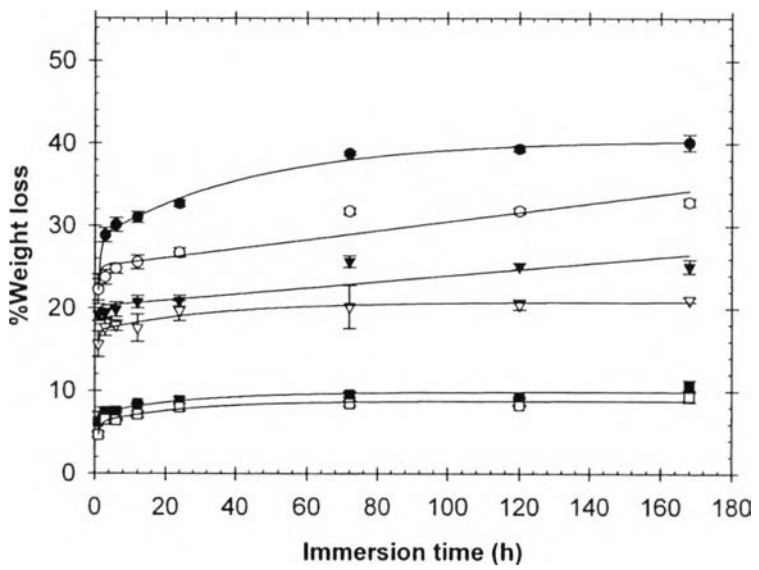


Figure 4.3 (continued).

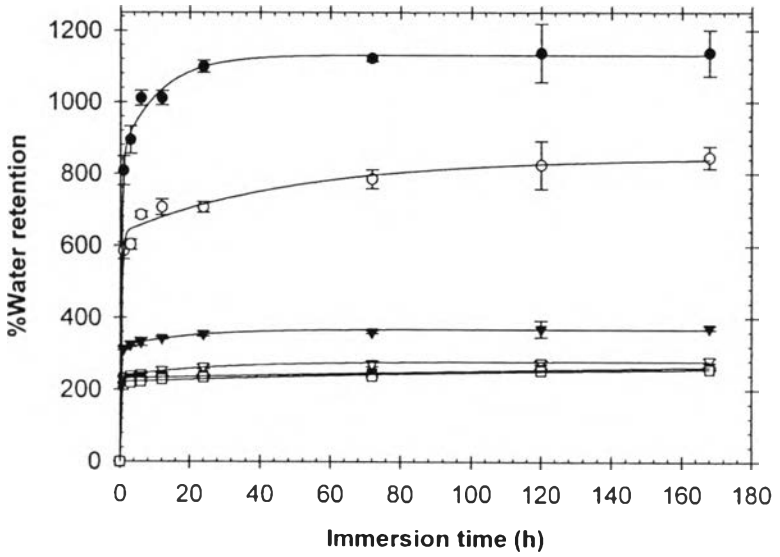


(a)

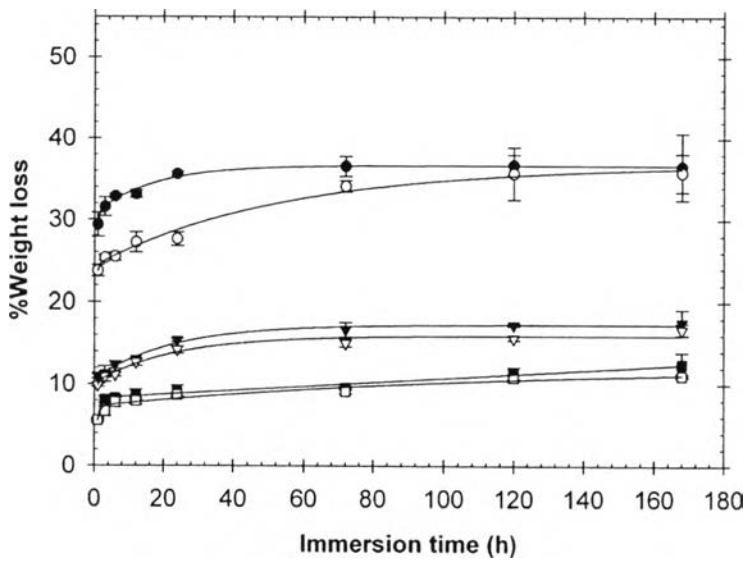


(b)

Figure 4.4 (a) Water retention and (b) weight loss behavior of cross-linked nAg-loaded gelatin hydrogel pads that had been immersed in acetate buffer (pH 5.5) at 32 °C for various time intervals (n = 3). Different data sets were for hydrogels that had been cross-linked with different glutaraldehyde (GTA) contents: ● 0.5, ○ 1, ▼ 3, ▽ 5, ■ 7 and □ 9 μl/ml.

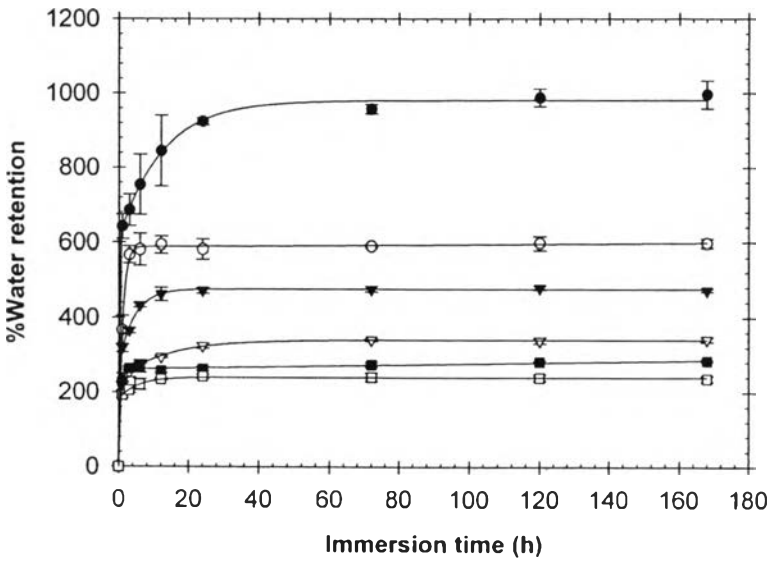


(a)

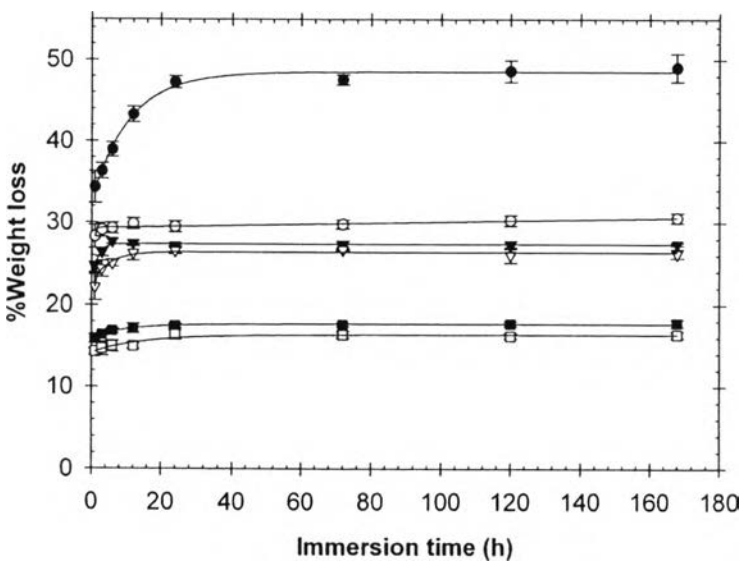


(b)

Figure 4.5 (a) Water retention and (b) weight loss behavior of cross-linked nAg-loaded gelatin hydrogel pads that had been immersed in distilled water (pH 6.5) at 37 °C for various time intervals (n = 3). Different data sets were for hydrogels that had been cross-linked with different GTA contents: ● 0.5, ○ 1, ▼ 3, ▽ 5, ■ 7 and □ 9 μl/ml.

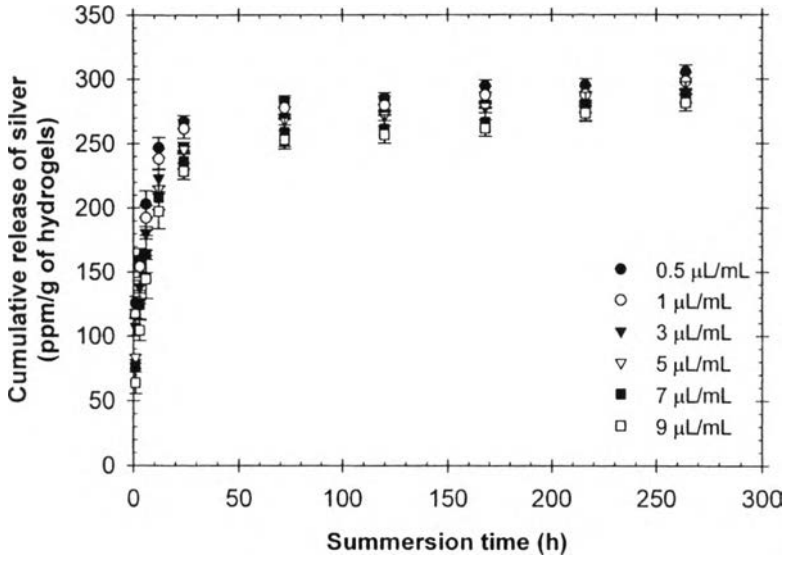


(a)

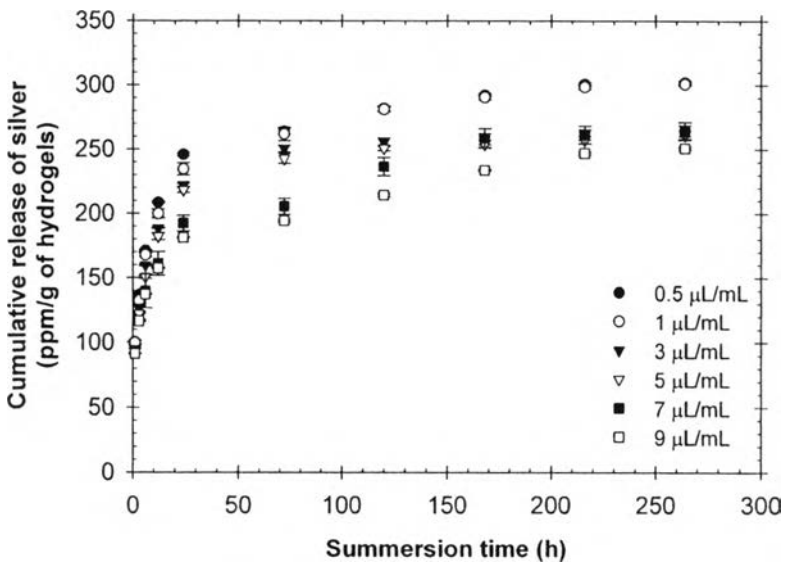


(b)

Figure 4.6 (a) Water retention and (b) weight loss behavior of cross-linked nAg-loaded gelatin hydrogel pads that had been immersed in simulated body fluid (SBF) buffer (pH 7.4) at 37 °C for various time intervals (n = 3). Different data sets were for hydrogels that had been cross-linked with different GTA contents: ● 0.5, ○ 1, ▼ 3, ▽ 5, ■ 7 and □ 9 μl/ml.

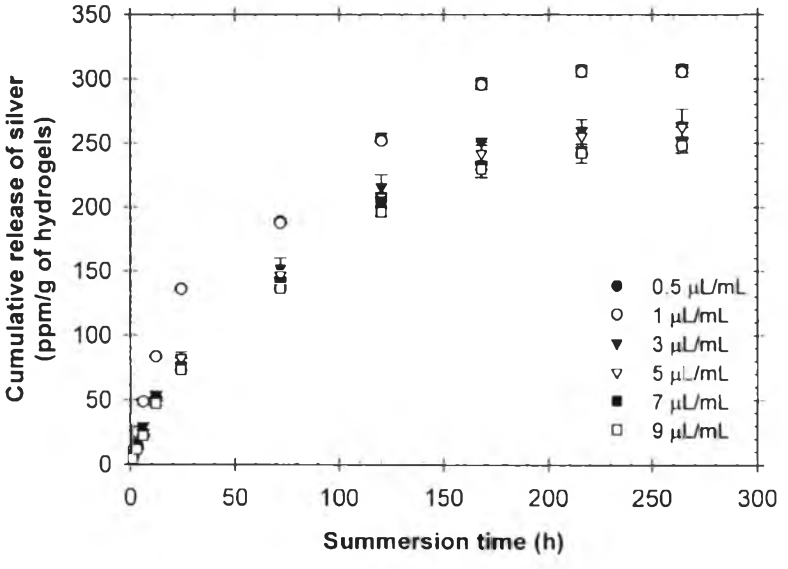


(a)



(b)

Figure 4.7 Cumulative release of silver from cross-linked nAg-loaded gelatin hydrogel pads that had been submerged in (a) acetate buffer (pH 5.5) at 32 °C, (b) distilled water (pH 6.5) at 37 °C and (c) SBF buffer (pH 7.4) at 37 °C for various time intervals (n = 3). Different data sets were for hydrogels that had been cross-linked with different GTA contents.



(c)

Figure 4.7 (continued).

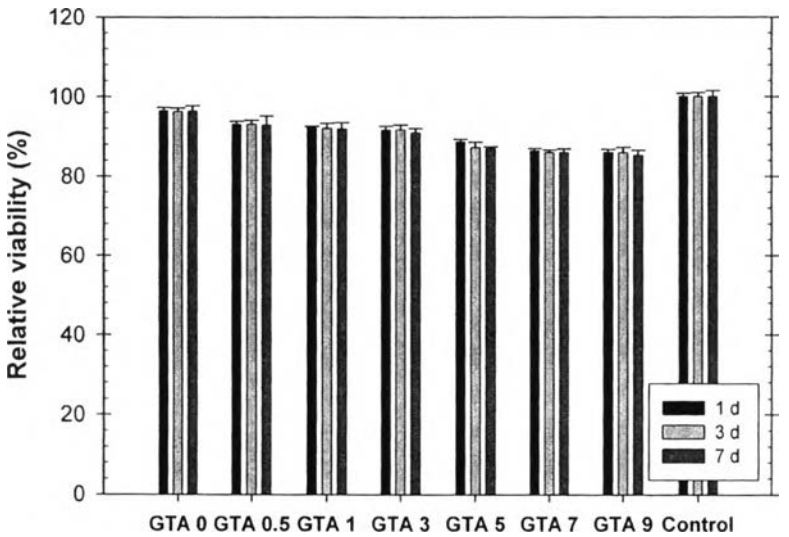


Figure 4.8 Indirect cytotoxicity evaluation of neat (i.e., GTA 0) and cross-linked gelatin hydrogel pads that had been cross-linked with GTA in different contents. The results were reported in terms of the viability of normal skin fibroblasts that had been cultured with extraction media obtained from the hydrogels in comparison with those cultured with fresh culture medium ($n = 3$). The extraction media were prepared by submerging the hydrogels in the cultured medium for various time intervals as indicated.

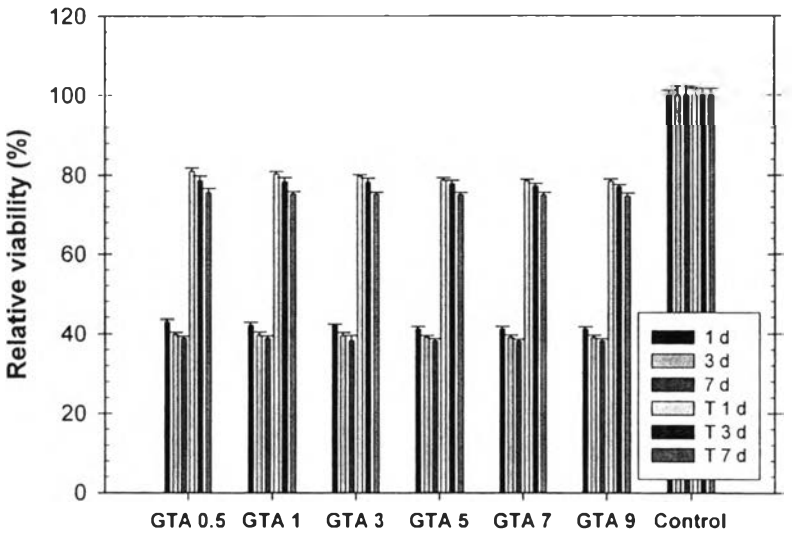


Figure 4.9 Indirect cytotoxicity evaluation of cross-linked nAg-loaded gelatin hydrogel pads without (i.e., 1 d, 3 d and 7 d) or with their subsequent treatment with a sodium metabisulfite solution (i.e., T 1 d, T 3 d and T 7 d). The results were reported in terms of the viability of normal skin fibroblasts that had been cultured with extraction media obtained from the hydrogels in comparison with those cultured with fresh culture medium (n = 3). The extraction media were prepared by submerging the hydrogels in the cultured medium for various time intervals as indicated.