# CHAPTER V
# IN VITRO EFFICACY AND TOXICOLOGY EVALUATION OF SILVER NANOPARTICLE-LOADED GELATIN HYDROGEL PADS AS ANTIBACTERIAL WOUND DRESSINGS

## 5.1 Abstract

The silver nanoparticle (nAg)-loaded gelatin hydrogel pads were prepared from 10 wt.-% gelatin aqueous solution containing silver nitrate ($AgNO_3$) at 0.75, 1.0, 1.5, 2.0 or 2.5 wt.-% by solvent-casting technique. The solutions were aged for various time intervals to allow for the formation of nAgs, the size of which increased with increasing $AgNO_3$ content in the solutions (i.e., from 7.7 to 10.8 nm, on average, for the solutions that had been aged for 15, 12, 8, 8 and 8 h, respectively). The hydrogels were cross-linked with a glutaraldehyde aqueous solution (50 wt.-%, at 1 $\mu l \cdot ml^{-1}$). At 24 h of submersion in phosphate buffer saline (PBS) or simulated body fluid buffer (SBF) solution, about 40.5-56.4 % or 44.4-79.6 % of the as-loaded amounts of silver was released. Based on the colony count method, these nAg-loaded hydrogels were effective against *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*, with at least 99.77 % bacterial growth inhibition. Unless they had been treated with a sodium metabisulfite aqueous solution, these hydrogels were proven, based on the indirect cytotoxicity evaluation, to be toxic to human's normal skin fibroblasts. Lastly, only the hydrogels that contained $AgNO_3$ at 0.75 and 1.0 wt.-% were not detrimental to the directly-cultured skin cells.

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

## 5.2 Introduction

Infections that develop in traumatic and surgical wounds remain a major problem. One key approach to minimize such a problem is the application of topical antimicrobial agents (Matsuda, 1992; Burkatovskaya, 2006). Silver has long been

recognized as a broad-spectrum and highly effective antimicrobial agent for treating infectious wounds (Klasen, 2000). In 1884, a German obstetrician, C.S.F. Crede, formulated 1 % silver nitrate ($AgNO_3$) in eye drops to treat gonococcal ophthalmia neonatorum, which could be the first scientifically-documented medical use of a silver-based compound (Russell, 1994). The use of silver-based dressings was shown to enhance epithelialization of clean wounds in pig models, indicating the beneficial effect of silver ions in wound care besides its antimicrobial activity (Geronemus, 1979; Lansdown, 1997). Laufman *et al.* (1989) reported that 0.5% $AgNO_3$ aqueous solution may be as efficacious as antibiotics for the prophylaxis of burn infections. Notwithstanding, $AgNO_3$ is hypotonic and can seriously cause hyponatremia and hypochloremia (Poon, 2004; Vlachou, 2007). Due to the reduction of silver ions, application of $AgNO_3$ can cause the color of the wound sites to change into dark gray or black, which can cause irritation (Lloyd, 1978; Qin, 2005; Hermans, 2006). To prevent these problems, much attention has been given to pure atomic silver, i.e., in the form of nanoparticles (hereafter, nAgs), which exhibits much stronger anti-microbial activity than the bulk silver metal (Cho, 2005). Several methods have been used to prepare nAgs from silver ions: they are, for instances, chemical reduction, γ-ray irradiation and ultrasonication (Wang, 2001; Zhang, 2001; Lu, 2003). Recently, a variety of water-soluble polymers such as poly(vinyl alcohol) (PVA), poly(vinyl pyrolidone) (PVP), poly(ethylene glycol) (PEG) (Luo, 2005), gum acacia (Yu, 2004; Dror, 2006), cellulose-based polymers (Kwon, 2005) and gelatin (Pal, 1997) have been used in the synthesis of nAgs, due mainly to their abilities to act as both the reducing agents and stabilizers.

Gelatin is a biopolymer derived from collagen via either acid or alkaline hydrolysis and, as such, it is biodegradable, biocompatible (Ward, 1997), non-toxic (Li, 1998; Ugwoke, 1998), naturally abundant and cheap. It is widely used in pharmaceutical and biomedical fields as encapsulating shells of capsules, carriers for therapeutic agents, sealants for vascular prostheses, wound dressings and absorbing pads for surgical uses (Hastings, 1984; Rose, 1987; Digenis, 1994; Esposito, 1995; Otani, 1998). Hydrogels, due to their ability to imbibe large quantities of water and other aqueous media, are flexible, with properties that can be adjusted to resemble those of skin. Thus, their proposed uses have been in areas such as drug delivery

devices, contact lens, cell transplantation matrices and wound dressings (Rathna, 1996). As wound dressings, they should be able to maintain optimal amount of exudates that keeps the wound in a moist environment.

Based on the above-mentioned reasons, it is of our interests to prepare nAg-loaded gelatin hydrogels from gelatin and $AgNO_3$ to be used as antibacterial wound dressings. In our previous publication (Rattanaruengsrikul, 2009), nAg-loaded gelatin hydrogel pads were prepared from 10 wt.-% gelatin solution containing 2.5 wt.-% $AgNO_3$ in 70 % v/v acetic acid by solvent-casting technique. The formation of nAgs was achieved upon aging the $AgNO_3$-containing gelatin solution under mechanical stirring for various time intervals, which was monitored by UV-visible spectrometry and examined by transmission electron microscopy. Upon aging for 4-7 d, the size of these particles ranged between 9-28 nm. To improve the water resistance of the hydrogels, various amounts of glutaraldehyde (GTA) were added to the $AgNO_3$-containing gelatin solution to cross-link the obtained gelatin hydrogels. With an increase in the GTA content used to cross-link the hydrogels, the water retention, the weight loss, and the cumulative released amounts of silver were found to decrease. All of the nAg-loaded gelatin hydrogels could inhibit the growth of Gram-negative *Escherichia coli* and Gram-positive *Staphylococcus aureus*, however, based on an indirect cytotoxicity evaluation, they showed toxicity towards normal human fibroblasts.

The toxicity of the nAg-loaded gelatin hydrogel pads was hypothesized to be a result of the presence of $NO_3^-$ ions, which, upon ionic exchange with the less toxic metabisulfite anions, the hydrogels became less toxic to the fibroblastic cells (Rattanaruengsrikul, 2009). The present contribution investigated further the effect of the initial concentration of $AgNO_3$ that would be loaded in the gelatin solution to finally obtain the nAg-loaded gelatin hydrogel pads. The major aim was to find an optimized concentration of $AgNO_3$ in the gelatin solution that resulted in the hydrogels with appropriated antibacterial activity against *E. coli* (Gram-negative), *Pseudomonas aeruginosa* (Gram-negative) and *S. aureus* (Gram-positive) and less toxicity towards the fibroblastic cells.

## 5.3 Experimental

### 5.3.1 Materials

Gelatin (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). Saturated glutaraldehyde aqueous solution (GTA; 5.6 M or 50 wt.-% 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). All chemicals were of analytical reagent grades and used without further purification.

### 5.3.2 Sample Preparation

#### *5.3.2.1 Preparation of nAg-Containing Gelatin Solutions*

The base gelatin solution was prepared at a fixed concentration of 10 wt.-%, simply by dissolving gelatin powder in distilled water under mechanical stirring at 40 °C until the mixture became a clear solution. $AgNO_3$ at various concentrations (0.75, 1.0, 1.5, 2.0 and 2.5 % by weight of gelatin powder) was then added slowly in the gelatin solution. The $AgNO_3$-containing gelatin solutions were then aged at various time intervals under mechanical stirring at 40 °C to allow the formation of nAgs within these solutions.

#### *5.3.2.2 Preparation of nAg-Loaded Gelatin Hydrogel Pads*

GTA at a fixed loading of 1 $\mu l \cdot ml^{-1}$ of the gelatin solution was mixed with each of the $AgNO_3$-containing gelatin solutions that had been aged for a proper time interval under mechanical stirring at 40 °C 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. The film castings were treated further in an oven at 120 °C for 4 d to maximize the cross-linking reaction. To minimize the toxicity effect from the $NO_3^-$ ions, the cross-linked nAg-loaded gelatin hydrogel pads were immersed in 0.4 % w/v sodium metabisulfite aqueous solution (Vandervoort, 2004; Rattanaruengsrikul, 2009) for 24 h, rapidly washed four times in distilled water and, finally, air-dried at ambient condition (temperature = 25 ± 2 °C and RH = 65 ± 5 %)

for 24 h. The thicknesses of the cross-linked nAg-loaded gelatin hydrogel pads in their dry state ranged between 150 and 170 μm.

5.3.3 Characterization

*5.3.3.1 Formation of nAgs*

The formation of nAgs in the $AgNO_3$-containing gelatin solutions that had been aged for various time intervals was evaluated from the spectra of the surface plasmon band located at the wavelengths of about 416-445 nm (Arai, 2001; Yang, 2003; Frattini, 2005; Lee, 2005), using a Shimadzu UV-2550 UV-visible spectrophotometer. The shape and the size of the as-formed nAgs along with its distribution were characterized by a JEOL JEM-2100 transmission electron microscope (TEM). The size of the nAgs was measured from the TEM images on at least 50 individual particles, using a SemAfore software.

*5.3.3.2 Preparation of Media*

*5.3.3.2.1 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 ℃ 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.

*5.3.3.2.2 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.

*5.3.3.3 Release Characteristics of Silver*

Prior to the release assay, the actual amounts of silver (either in the form of $Ag^+$ ions or nAgs) in the uncross-linked nAg-loaded gelatin hydrogel pads (circular disc; 15 mm in diameter) were determined. The quantification was carried out by dissolving the hydrogel specimens in 5 ml of 95 % nitric acid ($HNO_3$), followed by the addition of simulated body fluid buffer solution (SBF; pH 7.4) to attain a total volume of 50 ml. After that, each of the silver-containing solutions was quantified for the amount of silver by a Varian SpectrAA-300 atomic absorption spectroscope (AAS). The experiments were carried out in triplicate. The release of silver from the cross-linked nAg-loaded gelatin hydrogel pad specimens (circular disc; 15 mm in diameter) was assessed in 50 ml of either phosphate buffer saline solution (PBS; pH = 7.4) or SBF at 37 °C for various time intervals (i.e., 1, 3, 6, 12, 24, 72, 120, 168, 216, 264, 312 and 360 h). At each releasing time point, the medium was totally removed and an equal amount of the fresh medium was replaced. The amounts of the released silver in the withdrawn media (i.e., sample solutions) were also determined by AAS. At each time point, the measurements were carried out in triplicate. The obtained data were carefully calculated to obtain the cumulative amounts of silver released from the hydrogel specimens. The cumulative release profiles of silver were expressed based on the unit weight of the specimens.

*5.3.3.4 Antibacterial Evaluation*

Antimicrobial activity of the nAg-loaded gelatin hydrogel pads was tested against Gram-positive *Staphylococcus aureus* (*S. aureus*, ATCC 6538), Gram-negative *Escherichia coli* (*E. coli*, ATCC 25922) and Gram-negative *Pseudomonas aeruginosa* (*P. aeruginosa*, ATCC 27853), using 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:

$$\text{Bacterial growth inhibition (\%)} = (B - A)/B \times 100, \qquad (1)$$

where $A$ and $B$ are the 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.

*5.3.3.5 Culture of Fibroblastic Cells*

Human's normal skin fibroblasts were 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)] at 37 °C in a humidified atmosphere containing 5% $CO_2$. The medium was changed twice a week. When the cultures reached 90% confluence, the cells were trypsinized [0.25 % trypsin that contained 1 mM of ethylenediaminetetraacetic acid (Invitrogen Corp., USA)], re-suspended in the culture medium and counted by a hemocytometer (Hausser Scientific, USA).

*5.3.3.6 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 ($10^{th}$ to $15^{th}$ passages) as reference. The nAg-loaded gelatin hydrogels were cut into circular disc specimens (about 15 mm in diameter) and these specimens were sterilized in 70 vol.-% ethanol aqueous solution for 30 min. Extraction media were prepared by immersing the specimens in serum-free medium (SFM; containing DMEM, 1 % L-glutamine, 1% lactalbumin, and 1 % antibiotic and antimycotic formulation) at the ratio between the weight of the specimens and the volume of SFM of 10 $mg \cdot ml^{-1}$ for 1, 3 and 7 d, respectively. Normal skin fibroblasts from the culture were seeded at a density of $1 \times 10^4$ cells/well in wells of a 96-well tissue-culture polystyrene plate (TCPS; Biokom Systems, Poland) and cultured for 16 h at 37 °C in an incubator to allow cell attachment on the surface of the plate. The culture medium was replaced with SFM for 3 h and replaced again with fresh-SFM and incubated further for 24 h. After that, the SFM medium was replaced with an extraction medium and the cells were re-incubated for 24 h. Finally, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Vandervoort, 2004) was used to quantify the viability of the cells. The viability of the cells cultured by fresh SFM was used as control.

*5.3.3.7 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 $1.5 \times 10^4$ 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.

*5.3.3.9 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.

## 5.4 Results and Discussion

### 5.4.1 Formation of nAgs in $AgNO_3$-Containing Gelatin Solutions

The gelatin solutions containing different amounts of silver nitrate ($AgNO_3$) were first aged for various time intervals under mechanical stirring to allow for the formation of silver nanoparticles (nAgs). The formation of nAgs in the $AgNO_3$-containing gelatin solutions after they had been aged for various time intervals can be easily recognized from the change in the color of the solutions and from the change in the UV-visible absorption spectra. The color of the $AgNO_3$-containing gelatin solutions would change from transparent yellow to dark brown, the extent of which depended on the concentration of the as-formed nAgs as $Ag^+$ ions were reduced to nano-crystalline, metallic $Ag^0$. As a result, the brown color of the nAg-containing gelatin solutions became increasingly darker with an increase in the aging time interval. Furthermore, the brown color of the nAg-loaded gelatin solutions, at a given aging time interval, became darker with an increase in the initial amount of the as-loaded $AgNO_3$.

Figure 5.1 shows spectrophotometrical evidence for the formation of nAgs in the gelatin solutions containing $AgNO_3$ at 0.75, 1.0, 1.5, 2.0 and 2.5 wt.-% after they had been aged for various time intervals. It has been shown in the literature that the formation of nAgs can be observed from the appearance of the surface plasmon resonance absorption peak that centers around 416-445 nm in the UV spectrum (Arai, 2001; Yang, 2003; Frattini, 2005; Lee, 2005). Such peaks for the gelatin solutions containing $AgNO_3$ at 0.75, 1.0, 1.5, 2.0 and 2.5 wt.-%, after aging, were first observed at 12, 10, 7, 7 and 7 h, respectively. These results implied that the formation nAgs in the $AgNO_3$-containing gelatin solutions occurred relatively faster when the $AgNO_3$ concentration in the solutions was greater. The gelatin solutions containing $AgNO_3$ at 0.75 and 1.0 wt.-% exhibited the absorption peaks over the wavelengths of around 416-429 nm, while those at higher loadings (i.e., 1.5, 2.0 and 2.5 wt.-%) showed the peaks over a wider range of 416-437 nm. Moreover, the intensity of the peaks, for any given loading of $AgNO_3$, increased, while the position of the peaks shifted towards a greater wavelength, with an increase in the time used in the aging of the solutions. These results related well with the change in the color of the $AgNO_3$-containing gelatin solutions that became darker brown with increases in both the aging time interval and the initial $AgNO_3$ concentration. While the increase in the peak intensity should relate to the increase in the number of the as-formed nAgs, the shift in the peak positions towards higher wavelengths should relate to the increase in the size of the as-formed particles.

In our previous work (Vandervoort, 2004), noticeable change in the UV spectra was observed when the $AgNO_3$-containing gelatin solution had been aged for at least 2 d. In the present work, noticeable change in the UV spectra for all of the $AgNO_3$-containing gelatin solutions was observed just in the matter of hours after aging. The discrepancy from the previous work could be due to the type of solvent used (viz. distilled water was used as the solvent here as opposed to 70% v/v acetic acid that was used in the previous work (Vandervoort, 2004)). The presence of both the acetate anions and protons could help to stabilize $Ag^+$ ions in the aqueous phase, while, in distilled water, the reduction of $Ag^+$ ions by certain functionalities of gelatin molecules should be more energetically favorable than their hydration state in the medium. In the previous work (Vandervoort, 2004), the $AgNO_3$-containing

gelatin solution that had been aged for 5 d was chosen for further investigation, as, at this aging time point, the amount of the as-formed nAgs was high enough. At such the aging time point, the intensity of the surface plasmon resonance absorption peak was about 1.4 a.u. Therefore, in the present work, the aging time points that resulted in the $AgNO_3$-containing gelatin solutions exhibiting the intensities of the respective absorption peaks of about 1.4 a.u. were chosen. Such aging time points for the gelatin solutions containing $AgNO_3$ at 0.75, 1.0, 1.5, 2.0 and 2.5 wt.-% were 15, 12, 8, 8 and 8 h, respectively (see Figure 5.1). The shape and size of the nAgs generated in these solutions were studied by TEM and the representative images are shown in Figure 5.2. These images clearly show the existence of nAgs and the size of these particles increased with an increase in the initial $AgNO_3$ content in the solutions (i.e., 7.7 ± 2.7, 8.7 ± 2.1, 9.0 ± 2.8, 9.9 ± 2.5 and 10.8 ± 3.3 nm for the gelatin solutions containing $AgNO_3$ at 0.75, 1.0, 1.5, 2.0 and 2.5 wt.-%, respectively), despite the hypothetical decrease in the aging time interval. The increase in the size of these particles should be a direct result of the increased tendency for nucleation.

Based on the results shown in Figures 5.1 and 5.2, it is obvious that gelatin is an effective reducing agent for $Ag^+$ ions. As already pointed out by us (Vandervoort, 2004), the formation of $Ag^0$ nuclei should originate from the complexation of $Ag^+$ ions to some amino acid residues in gelatin that contain either carboxylic acid or neutral amino groups (Zhang, 2001; Lu, 2003). These amino acid residues are glutamic acid, aspartic acid and arginine, which accounted to about 24% of the dry weight of the protein (Songchotikunpan, 2008).

### 5.4.2 Characterization of nAg-Loaded Gelatin Hydrogel Pads

#### *5.4.2.1 Release Characteristics of Silver*

Without cross-linking, a solvent-cast gelatin film dissolves readily in an aqueous medium. To impart the water resistance to the as-prepared nAg-loaded gelatin hydrogel pads, a glutaraldehyde (GTA) aqueous solution was used as the cross-linking reagent. This is simply because of the effectiveness of GTA as a cross-linking agent for gelatin and (Zhang, 2006; Songchotikunpan, 2008), particularly, its inexpensiveness. Previously (Vandervoort, 2004), we have shown that an increase in the GTA content used to cross-link the nAg-loaded gelatin

hydrogels caused the water retention and the loss in the weight in an aqueous medium to decrease, the cross-link density to increase, the cumulative amount of the released silver to slightly decrease, and the yield strength in the wet state to decrease. Based on these results, the GTA content used to cross-link the nAg-loaded gelatin hydrogels in the present studies was fixed at 1 $\mu l \cdot ml^{-1}$. At this particular content, the resulting gelatin hydrogels could absorb large quantities of water upon submersion in an aqueous medium, while being able to withstand reasonably high stresses (Vandervoort, 2004). Figure 5.3 shows representative photographic images of the neat and the nAg-loaded (viz. the initial $AgNO_3$ content in the $AgNO_3$-containing gelatin solution was 2.5 wt.-%) gelatin hydrogel pads that had been cross-linked with 1 $\mu l \cdot ml^{-1}$ GTA. Evidently, the color of the neat gelatin hydrogels was transparently yellow, while that of the nAg-loaded gelatin hydrogels was transparently brown. However, the shade of the brown color of the nAg-loaded gelatin hydrogels depended on the initial $AgNO_3$ contents in the $AgNO_3$-containing gelatin solutions as well as on the time intervals used to age the solutions.

Prior to investigating the release characteristics of silver from the cross-linked nAg-loaded gelatin hydrogel pads, the actual amounts of silver, either in the form of the free $Ag^+$ ions or the as-formed nAgs, in these hydrogels needed to be determined. For this purpose, the uncross-linked hydrogels that had been prepared from the gelatin solutions containing different amounts of $AgNO_3$ (i.e., 0.75, 1.0, 1.5, 2.0 and 2.5 wt.-%) were determined for the actual amounts of the as-loaded silver by first dissolving in 95% $HNO_3$, following by the addition of SBF. For the hydrogel pads that had been prepared from the gelatin solutions containing $AgNO_3$ at 0.75, 1.0, 1.5, 2.0 and 2.5 wt.-%, the theoretical amounts of the as-loaded silver in these hydrogels should be 4.73, 6.29, 9.38, 12.45 and 15.49 $mg \cdot g^{-1}$ of the hydrogels in their dry state, respectively. Upon dissolving these hydrogels in 50 ml of SBF/$HNO_3$ solution, the theoretical concentrations of the as-loaded silver in the medium should be 94.54, 125.74, 187.69, 249.02 and 309.76 $ppm \cdot g^{-1}$ of the hydrogels, respectively. These were determined experimentally to be 94.07 ± 2.41, 124.53 ± 0.42, 185.39 ± 1.06, 247.06 ± 2.76 and 307.32 ± 2.02 $ppm \cdot g^{-1}$ of the

hydrogels, respectively ($n$ = 3). These values were very close to the initial, theoretical values of silver originally loaded in the gelatin solutions.

Figure 5.4 illustrates the cumulative amounts of silver, either in the form of the free $Ag^+$ ions or the as-formed nAgs, that were released per gram of the cross-linked nAg-loaded gelatin hydrogel pads in either PBS or SBF as a function of submersion time. These hydrogel pads were prepared from the gelatin solutions containing $AgNO_3$ at 0.75, 1.0, 1.5, 2.0 and 2.5 wt.-%. In PBS, the release characteristics of silver from the hydrogels occurred in three stages. The first stage relates to the rapid, burst release of silver from the hydrogels within the first 24 h after submersion. At this time point, the cumulative amounts of silver released into the medium were 53.0, 58.9, 80.0, 112.4 and 124.5 $ppm \cdot g^{-1}$ of the hydrogels, which accounted to about 56.4, 47.3, 43.2, 45.5 and 40.5% of the as-loaded amounts of silver within the hydrogels. The second stage relates to the sustained release of silver from the hydrogels into the medium, which occurred between 24 to about 264 h after submersion. In the last stage, the cumulative amounts of silver in the medium leveled off to reach the final, maximum values of 93.6, 124.1, 184.0, 245.1 and 304.1 $ppm \cdot g^{-1}$ of the hydrogels, respectively. These values accounted to about 99 % of the as-loaded amounts of silver within the hydrogels. In SBF, the three stage-release characteristics of silver from the hydrogels were also observed. Similarly, the first stage occurred within the first 24 h, with the cumulative amounts of silver released into the medium being 74.9, 90.6, 126.0, 143.1 and 136.4 $ppm \cdot g^{-1}$ of the hydrogels. These values accounted to about 79.6, 72.7, 67.9, 57.9 and 44.4 % of the as-loaded amounts of silver within the hydrogels. The second stage, on the other hand, occurred between 24 to about 120 h (for the hydrogels that had been prepared from the gelatin solutions containing $AgNO_3$ at 0.75, 1.0 and 1.5 wt.-%) or about 216 h (for the hydrogels that had been prepared from the gelatin solutions containing $AgNO_3$ at 2.0 and 2.5 wt.-%). Finally, the cumulative amounts of silver in the medium leveled off to reach the final, maximum values of 94.5, 124.3, 185.3, 245.7 and 302.3 $ppm \cdot g^{-1}$ of the hydrogels. These values accounted to about 98 to 100% of the as-loaded amounts of silver within the hydrogels.

At any given submersion time point, the cumulative amounts of silver that had been released from the cross-linked nAg-loaded gelatin hydrogel pads into the SBF medium were greater than those of silver that had been released into the PBS medium. Even though the pH of both types of media was the same, the types and the amounts of anions that are present in each of the media were totally different. While PBS contains only a few types of anionic species, SBF contains a large number of them (see Supplementary data). The presence of the large number of anionic species in SBF may have contributed to the greater effusion of $Ag^+$ ions from the hydrogels.

*5.4.2.2 Antimicrobial Activity*

The antibacterial activity of silver-based materials is generally ascribed to four mechanisms (Klasen, 2000; Hermans, 2006; Atiyeh, 2007). Briefly, ionic silver ($Ag^+$) binds to the cell membrane, thus damaging it and/or interfering with various receptors; interferes with electron transport, thus impeding the production of adenosine triphosphate, the cell's energy "currency"; binds to DNA materials, thus impairing the ability of the cell to replicate; and causes the formation of insoluble compounds with certain nucleotides, proteins and/or the amino acid histidine, thus making them unavailable as intracellular "building blocks" (Klasen, 2000; Hermans, 2006; Atiyeh, 2007). Here, the antibacterial activities of the cross-linked nAg-loaded gelatin hydrogel pads were assessed against three common pathogenic bacteria, namely, *S. aureus*, *E. coli* and *P. aeruginosa*, based on the colony count method.

Figure 5.5 shows photographic images illustrating the bacterial colonies on agar plates that had been smeared with washing solutions from the neat and the nAg-loaded gelatin hydrogel specimens. The results shown in the figure were for the specimens that had been treated with 0.4% w/v sodium metabisulfite aqueous solution, while those for the untreated specimens were not shown. The treatment with the sodium metabisulfite aqueous solution was to reduce the toxicity of the nAg-loaded gelatin hydrogels (Vandervoort, 2004). The results, such as those shown in Figure 5, were used to calculate the bacterial growth inhibition values for all of the tested conditions. These values are summarized in Table 1. From the figure, it is obvious that the numbers of bacterial colonies of all of

the nAg-loaded gelatin hydrogels, for any given pathogenic bacterium, were significantly lower than that observed for the neat gelatin hydrogels. Among the different nAg-loaded gelatin hydrogels, the number of bacterial colonies, for any given type of bacteria, decreased with an increase in the amount of $AgNO_3$ originally loaded in the gelatin solution. This corresponds to the observed increase in the growth inhibition of the hydrogels with an increase in the initial $AgNO_3$ amount in the gelatin solution (see Table 5.1). Comparatively, the antibacterial activities of these nAg-loaded gelatin hydrogels were most effective against *P. aeruginosa*, followed by *S. aureus* and *E. coli*, respectively, In addition, for all of the tested bacteria, the antibacterial activities of the hydrogels reduced slightly after having been treated with the sodium metabisulfite aqueous solution.

The increase in the antibacterial activities of the hydrogels with an increase in the amount of $AgNO_3$ initially loaded in the gelatin solution corresponded well with the observed increase in the cumulative amounts of silver released from the hydrogels into the tested media (see Figure 5.4). The slight reduction in the antibacterial activities of the hydrogels upon their treatment in the sodium metabisulfite aqueous solution could be due to the partial release of silver from the hydrogels during the submersion in the solution. This caused the actual amounts of silver within the treated hydrogels to be slightly lower than those within the untreated ones, hence the reduction in the driving force for diffusion of silver from the treated hydrogels. Indeed, the amounts of silver released from the hydrogels into the sodium metabisulfite aqueous solution were quantified to be 19.3 ± 2.8, 21.6 ± 2.5, 20.9 ± 0.9, 25.7 ± 3.1 and 27.9 ± 3.4 $ppm \cdot g^{-1}$ of the hydrogels ($n$ = 3) (for the hydrogels that had been prepared from the gelatin solutions containing $AgNO_3$ at 0.75, 1.0, 1.5, 2.0 and 2.5 wt.-%, respectively). Lastly, literature reports showed that nAgs were effective against a number of common pathogenic microbes, including those used in the present contribution (Cho, 2005; Taylor, 2005; Son, 2006). Since nAgs were shown to be more effective against *P. aeruginosa* than *S. aureus* (Son, 2006) and they were shown to be more effective against *S. aureus* than *E. coli* (Cho, 2005), the results obtained in the present contribution were in good agreement with these studies.

*5.4.2.3 Indirect Cytotoxicity Evaluation*

To evaluate the applicability of the cross-linked nAg-loaded gelatin hydrogel pads as wound dressing materials, indirect cytotoxicity evaluation was conducted. Previously (Vandervoort, 2004), we showed that increasing both the GTA content used to cross-link the hydrogels and the incubation time used to prepare the extraction media from the crosslinked hydrogels resulted in a gradual and slight decrease in the relative viability of human's normal skin fibroblasts. As for the cross-linked nAg-loaded gelatin hydrogels that had been prepared from the gelatin solution containing $AgNO_3$ at 2.5 wt.-%, their toxicity towards the tested skin cells was postulated to be a result of the presence of the commonly-known toxic $NO_3^-$ ions (Atiyeh, 2007; Chen, 2008) and, upon the treatment with the sodium metabisulfite aqueous solution, the toxicity of the hydrogels was greatly reduced (Vandervoort, 2004). This was postulated to result from the ionic exchange of the nitrate anions with the less-toxic metabisulfite anions. Since, in the present contribution, the hydrogels were prepared at various initial concentrations of $AgNO_3$ (i.e., at 0.75, 1.0, 1.5, 2.0 and 2.5 wt.-%), it is still necessary to investigate how the variation in the amount of the as-loaded $AgNO_3$ affected to the toxicity of the resulting hydrogels.

Figure 5.6 illustrates the viability of the skin cells that had been cultured with the extraction media obtained from the nAg-loaded gelatin hydrogels in comparison with those cultured with the extraction media from the neat gelatin hydrogels (internal control) and the fresh culture medium (negative control) ($n$ = 3). Without the treatment with the sodium metabisulfite aqueous solution, the viability of the cells that had been cultured with the extraction media from the nAg-loaded gelatin hydrogels, regardless of the incubation time used in the preparation of the extraction media (i.e., 1, 3 or 7 d), was relatively low (39-43%, with respect to the that of the cells that had been cultured with the fresh medium). Note that the viability of the cells that had been cultured with the extraction media from the neat gelatin hydrogels was much greater and appeared to be comparable to the control (i.e., about 96%). Increases in both the initial content of $AgNO_3$ and the incubation time used to prepare the extraction media decrease the viability of the cultured cells only slightly. The results clearly showed that the incorporation of $AgNO_3$ of as low

as 0.75 wt.% in the gelatin solution used to prepare the hydrogels was detrimental to the skin cells. Upon the treatment with the sodium metabisulfite aqueous solution, the toxicity of the nAg-loaded gelatin hydrogels was immensely improved. Apparently, the viability of the cells cultured with the extraction media from the treated hydrogels decreased from about 92% down to about 75%, with increases in both the initial $AgNO_3$ content and the incubation time used to prepare the extraction media.

Hidalgo *et al.* (1998) and Hidalgo and Domínguez (1998) found that the use $AgNO_3$ at a high concentration inhibited both the proliferation and the DNA synthesis of the exposed human dermal fibroblasts, with less detrimental effect being observed at lower concentrations. Based on the results obtained, the treated hydrogels that had been prepared from gelatin solutions containing $AgNO_3$ at 0.75 and 1.0 wt.-% were chosen for further investigation.

*5.4.2.4 Attachment And Proliferation of Cultured Cells*

The applicability of the cross-linked nAg-loaded gelatin hydrogel pads as wound dressing materials was future assessed by observing the morphology of human's normal skin fibroblasts that had been seeded or cultured on their surfaces. The results were compared with those on the surfaces of the glass substrates (negative control), the neat gelatin hydrogel pads (internal, negative control) and the nAg-loaded gelatin hydrogel pads that had been prepared from the solution containing $AgNO_3$ at 1.5 wt.-% (positive control). The selected SEM images in two different magnifications, i.e., 500x and 1500x, of the seeded and the cultured cells are shown in Tables 5.2 and 5.3, respectively. At 5 h of cell seeding, a number of cells were able to attach on the glass and the neat gelatin hydrogel surfaces. An equal number of cells were also observed on the surfaces of the treated nAg-loaded hydrogels that had been prepared from the gelatin solutions containing $AgNO_3$ at 0.75 and 1.0 wt.-%. Evidently, the morphology of these attached cells was still round. Even though most of the cells on the surfaces of the treated nAg-loaded hydrogels showed evidence of early cytoplasmic expansion, in similar manner to all of the cells attached on the surfaces of the negative control substrates, some of them did not. This could implicate that the presence of silver, either in its ionic or nano-particulate form, and/or $NO_3^-$ adversely affected the cells. This was proven to be true

as the morphology of the cells attached on the treated nAg-loaded hydrogels that had been prepared from the gelatin solution containing $AgNO_3$ at 1.5 wt.-% was apparently abnormal, with the majority of them being round with no evidence of early cytoplasmic process.

The ability of the nAg-loaded gelatin hydrogels to support the attachment and the proliferation of the cells was further assessed on days 1 and 5, respectively, after the cells had been cultured on their surfaces. On day 1, the cells that had already been attached on the glass and the neat gelatin hydrogel substrates after the initial seeding period of 5 h extended their cytoplasm well over the surfaces, with evidence of cytoplasmic projections in the form of filopodia around the edge of the cells. Deposition of certain extra-cellular matrix (ECM) proteins was also observed. Similar result was observed for the treated nAg-loaded hydrogels that had been prepared from the gelatin solution containing $AgNO_3$ at 0.75 wt.-%. However, for the hydrogels from the solution containing $AgNO_3$ at 1.0 wt.-%, the cells' cytoplasmic bounderies were somewhat contracted, with less amount of the deposited ECM. Total detachment of the cells was observed for the cells that had been seeded and left to grow on the hydrogels that had been prepared from the gelatin solution containing $AgNO_3$ at 1.5 wt.-%. On day 5, the cells and their excreted ECM proteins covered almost all of the surfaces of both the glass and the neat gelatin hydrogel substrates and these cells appeared in their typical spindle-like morphology. For the treated nAg-loaded hydrogels that had been prepared from the gelatin solution containing $AgNO_3$ at 0.75 wt.-%, even though the amount of the deposited ECM was equivalent to that observed for the neat gelatin hydrogels, some of the cells were rounded up. The rounding-up of the cells was more evident for the treated nAg-loaded hydrogels that had been prepared from the gelatin solution containing $AgNO_3$ at 1.0 wt.-%. In addition, the amount of the deposited ECM was obviously lower. Again, no cells were observed on the hydrogels that had been prepared from the gelatin solution containing $AgNO_3$ at 1.5 wt.-%.

Hidalgo *et al.* (1998), Hidalgo and Domínguez (1998) and Poon and Burd (2004) all demonstrated that $AgNO_3$ imposed cytotoxic effect towards the cultured fibroblasts and/or keratinocytes in dose- and time-dependent manners. Hidalgo and Domínguez (1998) meticulously showed that the adverse

effect of $Ag^+$ ions to the cells was due to its ability to bind with various inter- and intra-cellular proteins, leading to the depletion of functional proteins that are used in the attachment, the proliferation and the differentiation of the cells. They also pointed out that the presence of $Ag^+$ ions was responsible for the detachment of the already-attached cells from a substrate (Domínguez, 1998). Nevertheless, it is suggested that the presence of various proteins in the wound exudates lessened the toxic effect of $Ag^+$ ions through the formation of protein-Ag complexes that reduced the bio-availability of the ions to the surrounding host cells (Hidalgo 1998; Hidalgo and Domínguez, 1998; Poon, 2004). Based on the results obtained in this contribution and those previously reported by others (Hidalgo 1998; Hidalgo and Domínguez, 1998; Poon, 2004), it is believed that the sodium metabisulfite-treated nAg-loaded gelatin hydrogel pads that had been prepared from the gelatin solution containing $AgNO_3$ at 0.75 and 1.0 wt.-% could be used as antibacterial wound dressing materials.

## 5.5 Conclusions

Gelatin hydrogel pads containing silver nanoparticles (nAgs) were prepared from aqueous gelatin solutions containing various amounts of silver nitrate ($AgNO_3$) of 0.75, 1.0, 1.5, 2.0 and 2.5 wt.-%. The size of the nAg particles that were formed in the gelatin solutions that had been aged for 15, 12, 8, 8 and 8 h, respectively, ranged between 7.7 and 10.8 nm on average. Almost all of the silver that had been loaded in the gelatin solutions was retained within the obtained gelatin hydrogel pads. Upon totally dissolving the hydrogels in 50 ml of simulated body fluid buffer (SBF)/nitric acid ($HNO_3$) solution, the concentrations of silver, either in the form of the free $Ag^+$ ions or the as-formed nAgs, within the obtained hydrogels were determined to be 94.1, 124.5, 185.4, 247.1 and 307.3 $ppm \cdot g^{-1}$ of the hydrogels, respectively, on average. Within the 24 h of submersion in the testing medium of either phosphate buffer saline solution (PBS) or SBF, either about 40.5-56.4 % or 44.4-79.6 % of the as-loaded amounts of silver within the hydrogels were able to release into either medium, respectively. Antibacterial activity of these hydrogels was tested against Gram-positive *Staphylococcus aureus*, Gram-negative *Escherichia coli* and Gram-

negative *Pseudomonas aeruginosa*, using the colony count method. After exposing the hydrogels to the microbial suspensions for 24 h, the numbers of the bacterial colonies were counted and it was found that the hydrogels, without or with the treatment with the sodium metabisulfite aqueous solution, could inhibit at least 99.77% of the bacterial growth. Without the treatment with the sodium metabisulfite aqueous solution, the hydrogels, even at the lowest concentration of the as-loaded silver within the hydrogels (i.e., about 0.75 wt.-%), were detrimental to human's normal skin fibroblasts. Upon the treatment with the sodium metabisulfite aqueous solution, the viability of the cells was greatly improved. Direct culture of the cells onto the hydrogels containing about 0.75, 1.0 and 1.5 wt.-% of the as-loaded silver showed that the greater the amount of the as-loaded silver, the higher the toxicity. At about 1.5 wt.-% of the as-loaded silver, total detachment of the cultured cells was evident.

## 5.6 Acknowledgments

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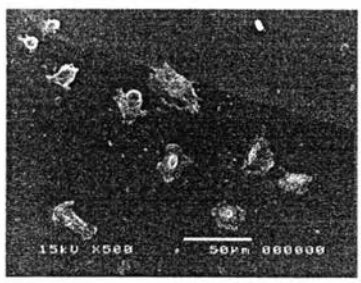
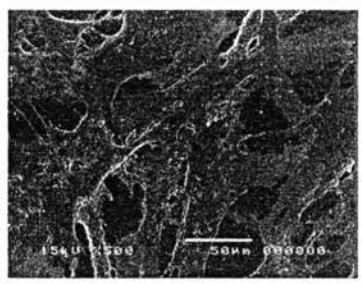
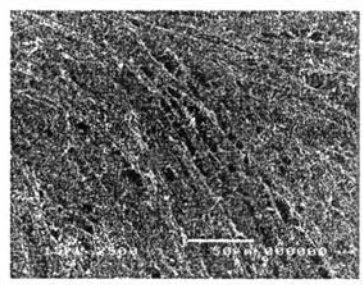
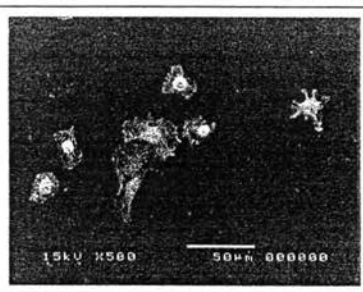
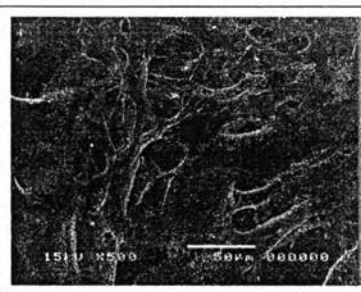
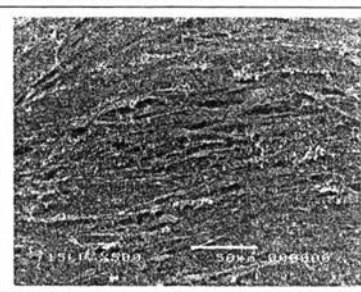
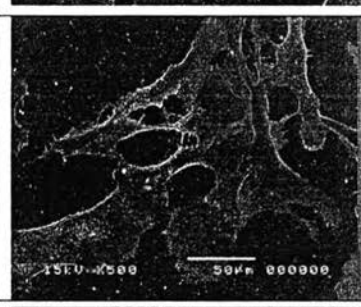
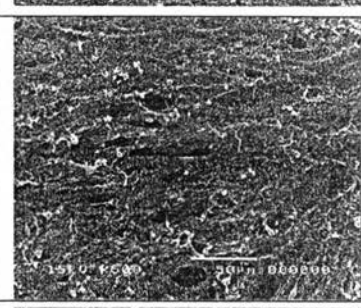
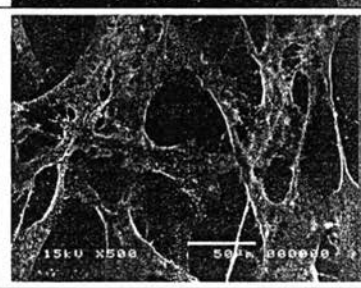
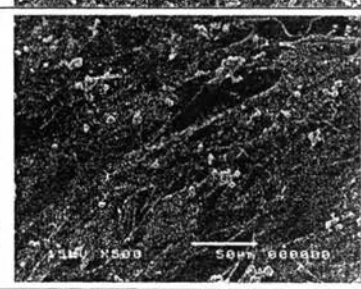
**Table 5.1** Bacterial growth inhibition (%) of the nAg-loaded gelatin hydrogel specimens without or with their subsequent treatment with 0.4% w/v sodium metabisulfite aqueous solution against *S. aureus*, *E. coli* and *P. aeruginosa* ($n = 3$)

| Bacteria | Bacterial growth inhibition (%) | | | | |
|---|---|---|---|---|---|
| | Without the treatment with 0.4% w/v sodium metabisulfite aqueous solution | | | | |
| | 0.75%$AgNO_3$ | 1%$AgNO_3$ | 1.5%$AgNO_3$ | 2%$AgNO_3$ | 2.5%$AgNO_3$ |
| *S. aureus* | 99.82 | 99.94 | 99.95 | 99.99 | 99.99 |
| *E. coli* | 99.80 | 99.91 | 99.92 | 99.95 | 99.98 |
| *P. aeruginosa* | 99.89 | 99.97 | 99.99 | 99.99 | 99.99 |
| **Bacteria** | **With the treatment with 0.4% w/v sodium metabisulfite aqueous solution** | | | | |
| | 0.75%$AgNO_3$ | 1%$AgNO_3$ | 1.5%$AgNO_3$ | 2%$AgNO_3$ | 2.5%$AgNO_3$ |
| *S. aureus* | 99.77 | 99.94 | 99.94 | 99.96 | 99.98 |
| *E. coli* | 99.67 | 99.84 | 99.87 | 99.91 | 99.97 |
| *P. aeruginosa* | 99.81 | 99.95 | 99.97 | 99.98 | 99.99 |

**Table 5.2** Representative, low-magnification SEM images (magnification = 500×; scale bar = 50 μm) of human's normal skin fibroblasts that had been cultured on surfaces of (a) glass slide, (b) the neat gelatin hydrogel specimens and the nAg-loaded gelatin hydrogel specimens that had been prepared from the solutions containing $AgNO_3$ at (c) 0.75, (d) 1.0 and (e) 1.5 wt.-% for different time intervals of 5 h, 1 d and 5 d, respectively

| Specimen | 5 h | 1 d | 5 d |
|---|---|---|---|
| (a) |  |  |  |
| (b) |  |  |  |
| (c) |  |  |  |
| (d) |  |  |  |

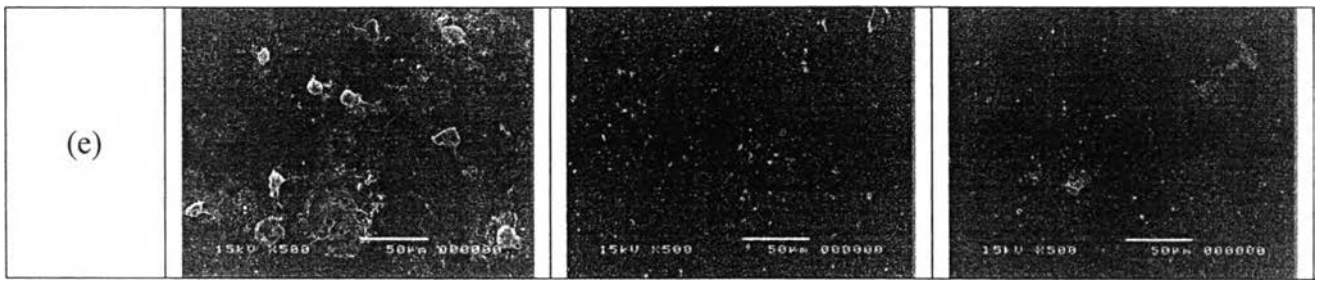
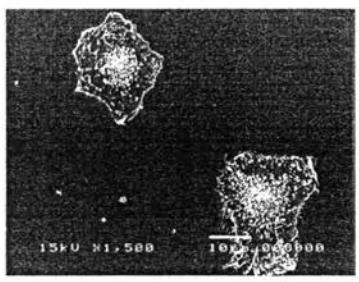
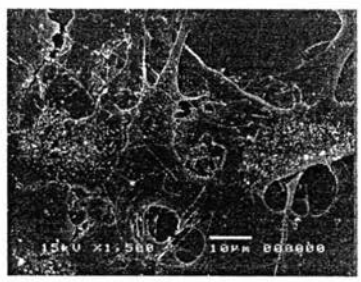
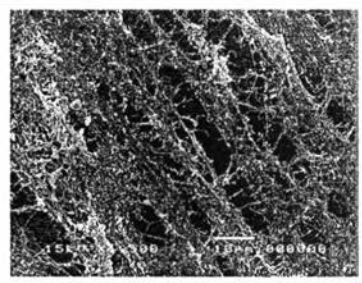
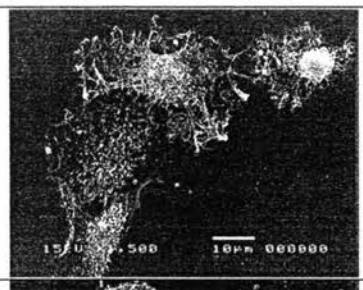
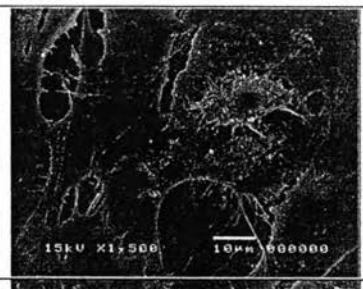
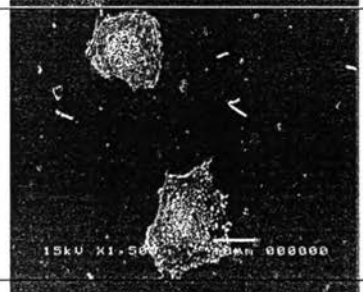
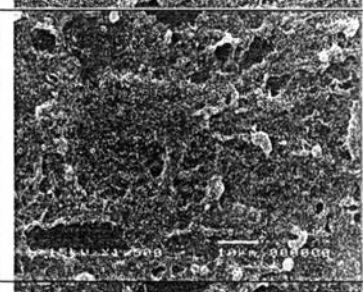
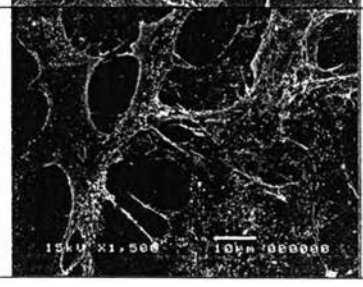
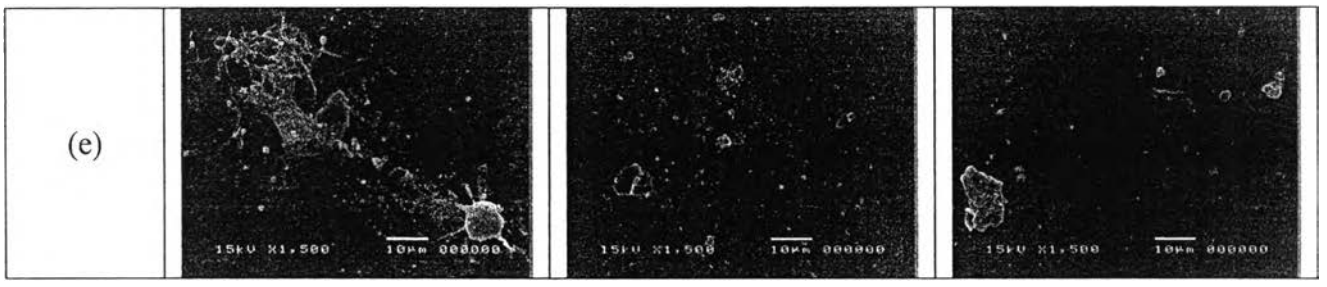

(e)
15kV X500 50µm 000000
15kV X500 50µm 000000
15kV X500 50µm 000000

**Table 5.3** Representative, high-magnification SEM images (magnification = 1500×; scale bar = 10 μm) of human's normal skin fibroblasts that had been cultured on surfaces of (a) glass slide, (b) the neat gelatin hydrogel specimens and the nAg-loaded gelatin hydrogel specimens that had been prepared from the solutions containing $AgNO_3$ at (c) 0.75, (d) 1.0 and (e) 1.5 wt.-% for different time intervals of 5 h, 1 d and 5 d, respectively

| Specimen | 5 h | 1 d | 5 d |
|---|---|---|---|
| (a) |  |  |  |
| (b) |  |  |  |
| (c) |  |  |  |
| (d) |  |  |  |


(e)
15kV X1,500
10µm 000000
15kV X1,500
10µm 000000
15kV X1,500
10µm 000000

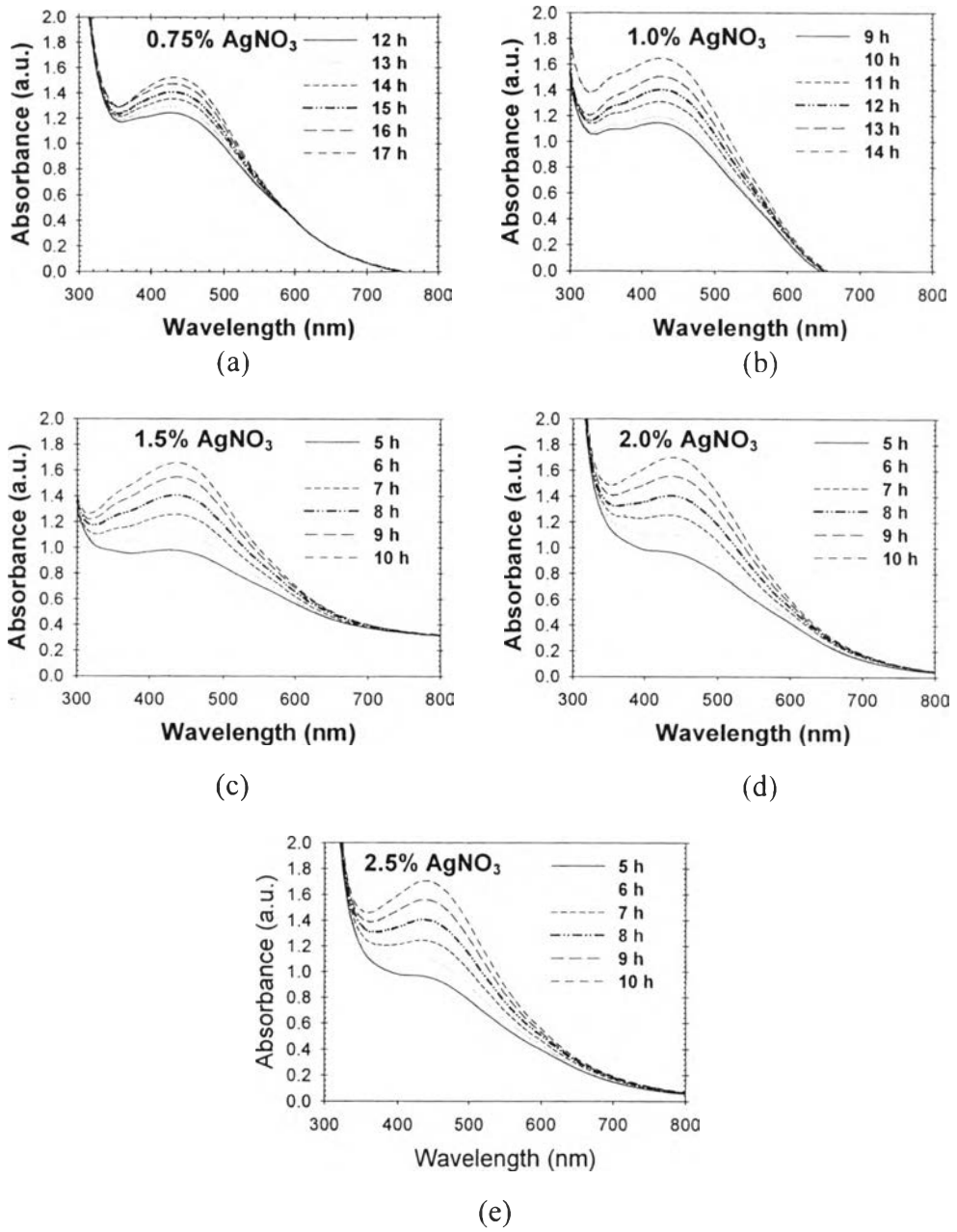



**Figure 5.1** UV-visible absorption spectra at various aging time intervals of the gelatin solutions that contained $AgNO_3$ at different loadings, i.e., (a) 0.75, (b) 1.0, (c) 1.5, (d) 2.0 and (e) 2.5 wt.-%. The $AgNO_3$-containing gelatin solutions that had been aged for 15, 12, 8, 8 and 8 h, respectively, were chosen for further fabrication into hydrogel pads for subsequent studies.

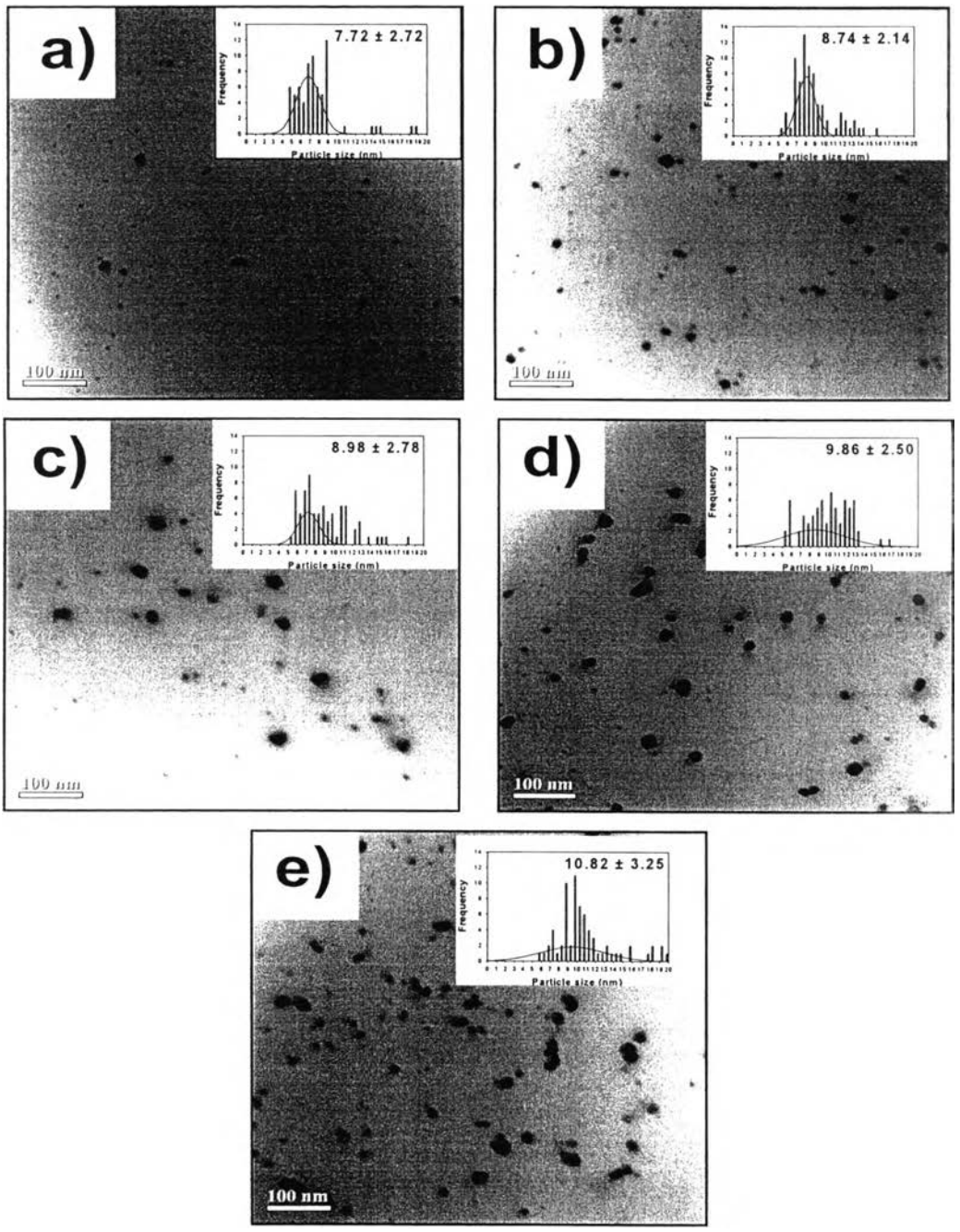



**Figure 5.2** Selected TEM images (magnification = 20,000x; scale bar = 100 nm) illustrating the morphology of silver nanoparticles (nAgs) that were formed in the gelatin solutions containing $AgNO_3$ at different loadings, i.e., (a) 0.75, (b) 1.0, (c) 1.5, (d) 2.0 and (e) 2.5 wt.-%. These solutions had been aged for 15, 12, 8, 8 and 8 h, respectively. The inset figures illustrate the size and its distribution of the as-formed nAgs.

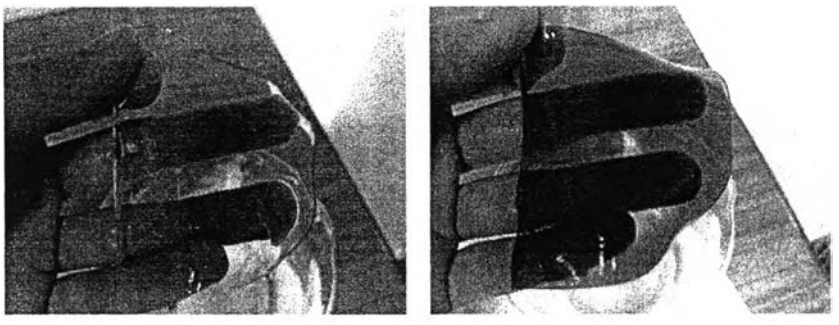
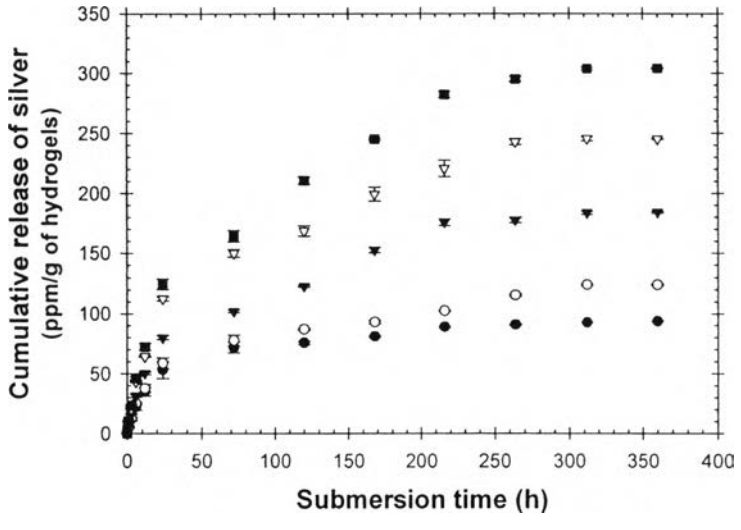
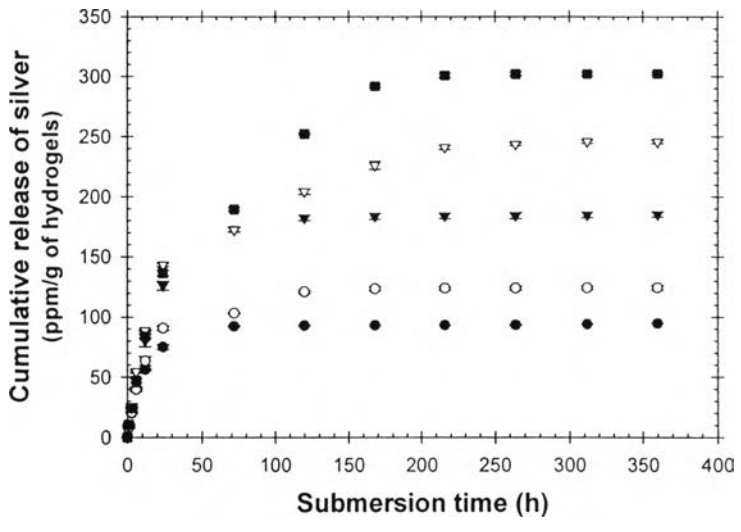



**Figure 5.3** Representative photographic images of (a) the neat and (b) the nAg-loaded gelatin hydrogel pads that had been cross-linked with 1 $\mu L \cdot mL^{-1}$ of 50 wt.-% glutaraldehyde aqueous solution (GTA). The nAg-loaded gelatin hydrogel pad was prepared from the solution containing $AgNO_3$ at 2.5 wt.-%.




(a)




(b)

**Figure 5.4** Cumulative release of silver from the cross-linked nAg-loaded gelatin hydrogel pads that had been submerged in (a) the phosphate buffer saline solution (PBS, pH 7.4) or (b) the simulated body fluid buffer solution (SBF, pH 7.4) at 37 °C for various time intervals ($n$ = 3). Different data sets were for the gelatin hydrogels that had been loaded with different $AgNO_3$ contents: (●) 0.75, (○) 1.0, (▼) 1.5, (▽) 2.0 and (■) 2.5 wt.-% and each of these hydrogels had been cross-linked with 1μl·ml$^{-1}$ of GTA.

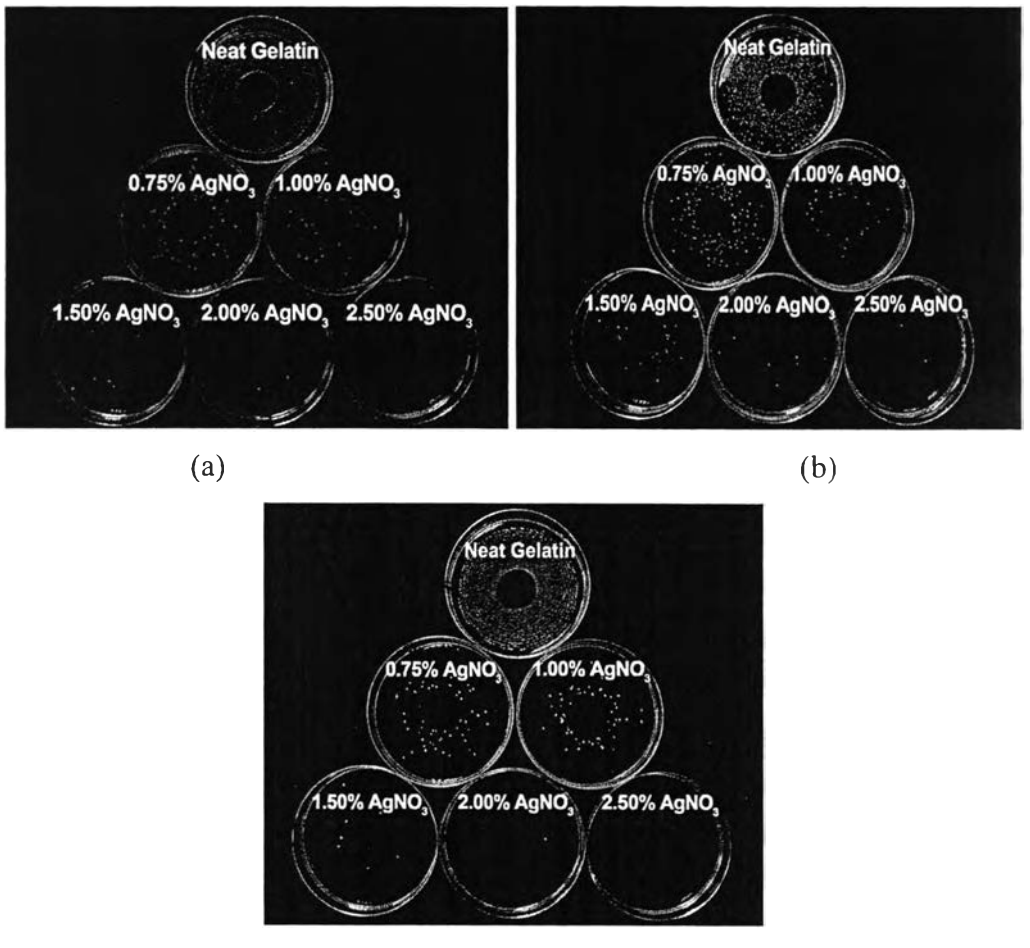
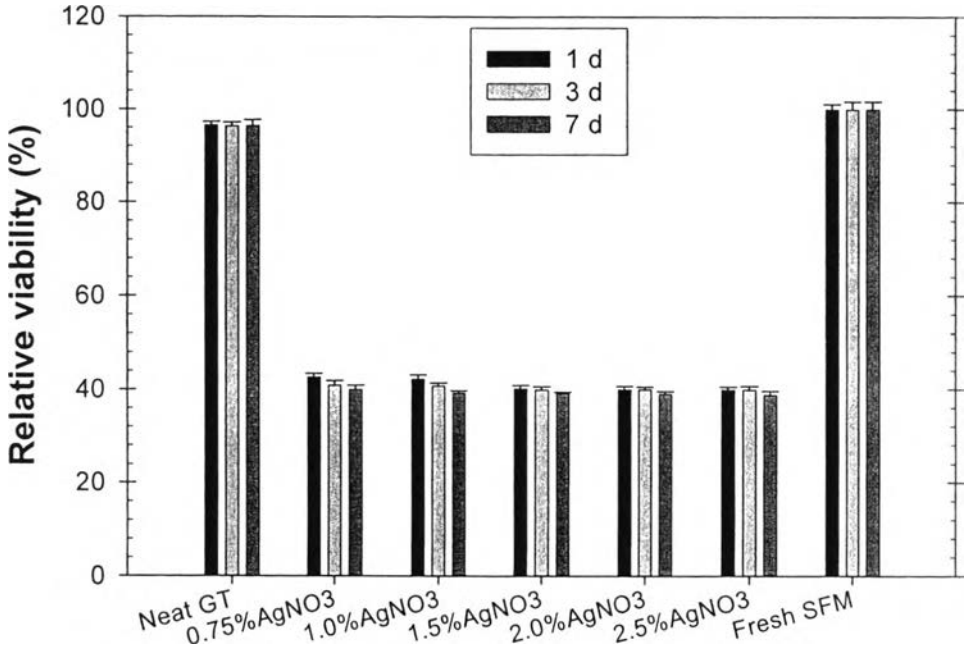
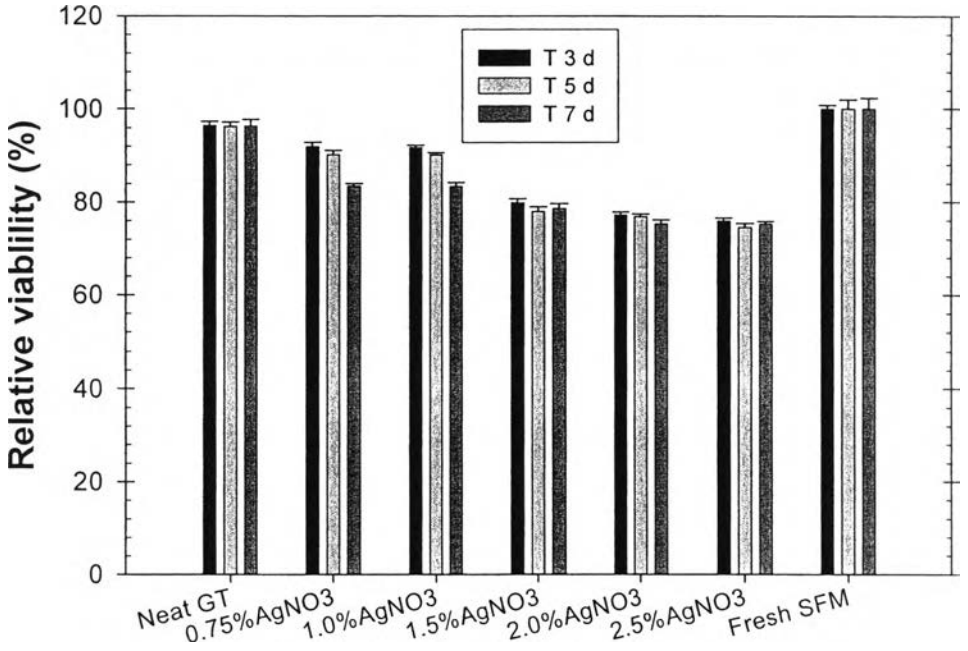



**Figure 5.5** Photographic images illustrating the bacterial colonies on agar plates that had been smeared with washing solutions from the neat and the nAg-loaded gelatin hydrogel specimens. Three types of bacteria were tested: (a) Gram-positive *Staphylococcus aureus*, (b) Gram-negative *Escherichia coli* and (c) Gram-negative *Pseudomonas aeruginosa*. Each of the hydrogel specimens was incubated with 1 ml of a respective bacterial suspension at 37 °C for 24 h and then washed in 100 mL of distilled water. Exactly 0.1 ml of the washing solution was finally plated on an agar plate. These hydrogel specimens had been treated in 0.4% w/v sodium metabisulfite aqueous solution prior to the assessment.




(a)

**Figure 5.6** Indirect cytotoxicity evaluation of the cross-linked gelatin hydrogel pads (a) without (i.e., 1, 3 and 7 d) or (b) with their subsequent treatment with 0.4% w/v sodium metabisulfite aqueous solution (i.e., T 1, T 3 and T 7 d). The results were reported in terms of the viability of human's normal skin fibroblasts that had been cultured with the extraction media obtained from the hydrogels in comparison with those cultured with the fresh culture medium ($n$ = 3). The extraction media were prepared by submerging the hydrogels in the culture medium for various time intervals as indicated.




(b)

**Figure 5.6** (continued)