CHAPTER V
IN VITRO STUDY OF ELECTROSPUN GELATIN FIBER MATS CONTAINING SILVER NANOPARTICLES WITH ANTI-BACTERIAL ACTIVITY

5.1 Abstract

Silver nanoparticles (nAg), a potent anti-bacterial agent, first appeared in the 0.75-2.00 wt.% $AgNO_3$-containing gelatin solution after it had been aged for at least 12 h, with the amount of nAg increasing with further increasing the aging time. Ultra-fine gelatin fiber mats with anti-bacterial activity against some common bacteria found on burn wounds were prepared from the gelatin solution (22% w/v in 70 vol.% acetic acid) containing 0.75-2.00 wt.% $AgNO_3$. Electrospinning of both the base and the 12 h-aged 0.75-2.00 wt.% $AgNO_3$-containing gelatin solutions resulted in the formation of smooth fibers, with the average diameters being 260, 248, 226, 215 and 206 nm, respectively. The average diameters of the as-formed nAg ranged between 10.18 and 12.80 nm. The nAg-containing gelatin fiber mats were further cross-linked with moist glutaraldehyde vapor to improve its stability in an aqueous medium. The weight loss and the water retention of the nAg-containing gelatin fiber mats in acetate buffer (pH 5.5) or simulated body fluid (SBF; pH 7.4) decreased with increasing the submersion time. The release of Ag^+ ions from 0.5 h-cross-linked nAg-containing gelatin fiber mats, by the total immersion method in the acetate buffer and (at the skin temperature of 32°C) occurred rapidly during the first 60 min and increased gradually afterwards, while that in SBF (at the physiological temperature of 37°C) occurred less than that of in acetate buffer. Zone of inhibition of these materials, regardless of the sample types, was the greatest against *Bacillus subtilis*, followed by *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli*, respectively. For quantitative evaluation with antibacterial activity of the 0.75-2.00% $AgNO_3$-loaded e-spun GT fibers mats, it is found that the percentage of reduction reached 99 % with all of bacteria, indicating that the silver particles are responsible for the antibacterial activity of the $AgNO_3$-loaded

electrospun gelatin fibers mats and this activity is quite strong. Finally, the potential for use of the 0.75-1.00% $AgNO_3$-loaded e-spun GT fiber mats as wound dressings was assessed by investigating the indirect cytotoxicity of these materials and the results showed that these materials posed no threat towards normal human dermal fibroblasts.

(Keywords: electrospinning; fibrous membrane; Ag nanoparticles; antimicrobial; wound dressing)

5.2 Introduction

A burn is an injury caused by heat, cold, electricity, chemicals, light, radiation, or friction. Burns can be highly variable in terms of the tissue affected, the severity, and resultant complications. Muscle, bone, blood vessel, and epidermal tissue can all be damaged with subsequent pain due to profound injury to nerve endings. Depending on the location affected and the degree of severity, a burn victim may experience a wide number of potentially fatal complications including shock, infection, electrolyte imbalance and respiratory distress. Patients with serious thermal injury require immediate specialized care in order to minimize morbidity and mortality. The survival rates for burn patients have improved substantially in the past few decades due to advances in modern medical care in specialized burn centers. Improved outcomes for severely burned patients have been attributed to medical advances in fluid resuscitation, nutritional support, pulmonary care, burn wound care, and infection control practices [1]. The use of topical chemotherapy has been fundamental in that regard and has helped to improve the survival of patients with major burns and to minimize the incidence of burn wound sepsis, a leading cause of mortality and morbidity in these patients [2].

One of the strategies that is gaining renewed attention for combating the threat of bacterial infection and preventing wound sepsis, is the use of noble metal antimicrobials the most prevalent of which is silver [3]. For centuries silver has been known to have bactericidal properties. As early as 1000 B.C., the antimicrobial properties of silver in rendering water potable were appreciated [4,5]. Silver

compounds have been exploited for their medicinal properties for centuries as well [6]. Interest in silver salts or silver salt solutions in the treatment of burn patients, however, completely disappeared around the Second World War [7]. It took many years for interest in silver (nitrate) to revive, under the stimulus of a publication by Moyer et al. [8]. At present, silver has reemerged as a viable treatment option for infections encountered in burns, open wounds, and chronic ulcers. Several products have incorporated silver for use as a topical antibacterial agent, such as silver nitrate, silver sulphadiazine (SSD) (Flammazine™, Smith & Nephew Healthcare Limited, Hull, Canada) [9], silver sulphadiazine/chlorhexidine (Silverex, Motiff Laboratories Pvt. Ltd. Kare Health specialties, Verna, Goa), SSD with cerium nitrate (Flammacerium, Solvay, Brussels, Belgium), and silver sulphadiazine impregnated lipidocolloid wound dressing Urgotul SSD (Laboratories Urgo, Chenove, France) [9-11]. In contrast to these silver agents, newly developed products such as Acticoat™ (Westaim Biomedical Inc., Fort Saskatchewan, Alberta, Canada) and Silverlon (Argentum Medical, L.L.C., Lakemont, Georgia), have a more controlled and prolonged release of nanocrystalline silver to the wound area. Another way is to convert silver ions to silver nanoparicles by using chemical reduction [12,13], UV irradiation [14], sonochemical deposition [15] and etc. Dressing material containing silver nanoparticles can sustain release of silver. Therefore, this mode of silver delivery allows the dressings to be changed with less frequency, thereby reducing risk of infection, cost of care, further tissue damage and patient discomfort [4],[16,17].

Electrospinning (e-spinning) is a process capable of producing fibers from materials of diverse origins, including polymers, with diameters in the nano- to micrometer range. A polymer liquid (i.e., melt or solution) is first loaded into a container with a small opening (used as the nozzle), and is then charged with a high electrical potential across a finite distance between the nozzle and a grounded collection device. When the electric field increases beyond a critical value – at which the repulsive electrical forces overcome the surface tension of the polymeric liquid droplet at the tip of the nozzle – a charged jet is ejected [18]. As the jet travels to the collector, it either cools down (in case of the melt) or the solvent evaporates (in case of the solution) to obtain ultrafine fibers in the form of a non-woven fabric on the

collector. The morphology of the electrospun (e-spun) fibers depends on a number of factors, such as solution properties (e.g., concentration, viscosity, conductivity, surface tension, etc.), processing conditions (e.g., electrical potential, collection distance, etc.), and ambient conditions (e.g., temperature, humidity, etc.) [19,20]. Some potential uses of e-spun fibers in biomedical fields are, for example, immobilization of enzymes [21], tissue-engineered scaffolds [22,23], and delivery carriers for DNA [24] and drugs [25-29].

Because of the inherent properties of gelatin such as natural abundance and inherent biodegradability in physiological environments and the unique characteristics of the e-spun fibers, e-spun gelatin fibers are ideal materials to be used as scaffolds for cell and tissue culture, carriers for topical/transdermal delivery of drugs, and wound dressings. In the present contribution, mats of gelatin fibers containing nAg were prepared by e-spinning with varying the amount of $AgNO_3$-loaded and these e-spun fiber mats were proposed to be used as wound dressing pads. The nAg-containing gelatin fiber mats were characterized for the release characteristic of the as-loaded silver as well as their anti-bacterial activity against some common bacteria found on burn wounds. Moreover, their biological properties were investigated by studying the cytotoxic effect of $AgNO_3$-loaded e-spun fibers and cell spreading of normal human dermal fibroblasts (NHDF) cells on material in vitro. Morphologies of the cells attached on the fibers were observed by scanning electron microscope (SEM).

5.3 Experimental

5.3.1 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). An aqueous solution of glutaraldehyde (GTA; 5.6 M or 50 vol.%) was purchased from Fluka (Switzerland). Sodium acetate, Sodium Chloride (NaCl), Sodium hydrogen carbonate ($NaHCO_3$), Potassium Chloride (KCl), Magnesium

Chloride ($MgCl_2.6H2O$), Magnesium Chloride ($MgCl_2.6H_2O$), Hydrochloric (HCl), Calcium Chloride ($CaCl_2$) and TRIS ($Na_2SO_4(CH_2OH)_3CNH_2$) were purchased from Ajax Chemicals, Australia. All chemicals were of analytical reagent grade and used without further purification.

5.3.2 Preparation and characterization of gelatin solutions containing nAg

Neat and $AgNO_3$-containing e-spun fiber mats were prepared as previously described [30]. Briefly, $AgNO_3$ was first dissolved in a quantity of 70:30 v/v glacial acetic acid/distilled water. A metered weight of gelatin powder was then added into the as-prepared $AgNO_3$ solution. Slight stirring was used to expedite the dissolution and homogenize the solution. The concentration of the base gelatin solution was fixed at 22% w/v (based on the volume of the mixed solvent) and the amount of $AgNO_3$ was varied at 0.75-2.00% w/w (based on the weight of the gelatin powder). To investigate the effect of aging time on the formation of nAg, the $AgNO_3$-containing gelatin solution was aged, while being stirred, for different time intervals.12 hr. of the aged $AgNO_3$-containing gelatin solutions were characterized for the shear viscosity and the conductivity using a Brookfield DV-III programmable rheometer and a SUNTEX SC-170 conductivity meter. The existence of the as-formed nAg in the as-aged $AgNO_3$-containing gelatin solutions was confirmed by monitoring the surface plasmon absorption band using a Shimadzu UV-2550 UV-visible spectrophotometer.

5.3.3 Preparation of neat and nAg-containing e-spun gelatin fiber mats

5.3.3.1 E-spinning

The base gelatin solution and the 0.75-2.00% $AgNO_3$-containing gelatin solution that had been aged for 12 hr were fabricated into neat and nAg-containing gelatin fiber mats by e-spinning. Firstly, each of the as-prepared solutions was loaded in a standard 10-mL glass syringe, the open end of which was attached with a blunt gauge-20 stainless steel hypodermic needle (OD = 0.91 mm), used as the nozzle. Both the syringe and the needle were tilted ~45°C from a horizontal baseline. A piece of aluminum (Al) sheet wrapped around a rotating cylinder (OD and width ≈ 15 cm; ~50-60 rpm) was used as the collecting device. A Gamma High-Voltage Research ES30P-5W DC power supply (Florida, USA) was

used to charge the solution by attaching the emitting electrode of positive polarity to the nozzle and the grounding one to the collecting device. A fixed electrical potential of 15 kV was applied across a fixed distance between the tip of the needle and the outer surface of the collecting device (i.e., collection distance, measured at right angle to the surface of the collecting device) of 20 cm. The e-spun fiber mats were collected continuously for 48 hr. The thickness of the neat and the nAg-containing gelatin fiber mats was measured by a Mitutoyo digital micrometer.

5.3.3.2 Cross-linking

Cross-linking of both the neat and the nAg-containing gelatin fiber mats was carried out by clamping each of the fiber mat samples between a pair of supporting stainless steel frames (4.5 cm × 10 cm) with adhesive tapes in a sealed chamber saturated with the vapor from 20 ml of the as-received GTA aqueous solution. The temperature of the chamber was maintained at 37°C and each fiber mat sample was exposed to the moist GTA vapor for 0.5 hr. After exposure, the sample was heat-treated in a heating oven at 110°C for 24 h to enhance the cross-linking reaction and to remove most, if not all, of the unreacted GTA.

To assess the extent of cross-linking, specimens from both the neat and the nAg-containing gelatin fiber mat samples (circular discs of ~1.5 cm in diameter) were weighed and then submerged in acetate buffer aqueous solution (pH 5.5, at the skin temperature of 32°C), or simulated body fluid (SBF; pH 7.4, at the physiological temperature of 37°C) for various submersion time intervals. The weight loss and the water retention of these specimens were determined according to the following equations:

$$\text{Weight loss (\%)} = \frac{M_i - M_d}{M_i} \times 100, \qquad (1)$$

$$\text{Water retention (\%)} = \frac{M - M_d}{M_d} \times 100, \qquad (2)$$

where M is the weight of each specimen after submersion in the medium at each submersion time point, M_d is the weight of the specimen in its dry state after submersion in the medium at each submersion time point, and M_i is the initial weight of the specimen in its dry state.

5.3.4 Characterization

The morphological appearance of both neat and nAg-containing gelatin fiber mats was observed by a scanning electron microscope (SEM; JEOL JSM-6400). The electrospun mats were sputtered with a thin layer of gold prior to SEM observation. Based on these SEM images, the average diameter of the as-spun fibers and average size of the beads (if any) could be measured. The results were reported as average values from at least 100 measurements. The neat and the nAg-containing gelatin fiber mats were examined either qualitatively or quantitatively for the as-formed nAg by a JEOL JEM-2100 transmission electron microscope (TEM). The average diameters of the as-formed nAg were determined from TEM images, using custom-code image analytical software. Mechanical integrity of the e-spun fiber mat samples with or without immersion in acetate buffer for 1 day was evaluated by a Lloyd LRX universal testing machine with 500 N load, 30 mm gauge length and 10 $mm \cdot min^{-1}$ cross-head speed at ambient conditions. The specimens of thickness about 160±20 μm were cut from the e-spun fiber mat samples (rectangular shape; 70 mm × 10 mm). At least 10 specimens for each sample type were tested.

5.3.5 Loading capacity and release characteristic of as-loaded silver

Prior to the release assay, the actual amount of silver (either in the form of elemental Ag^0 or Ag^+ ions) in the uncross-linked nAg-containing gelatin fiber mat specimens (circular disc; 1.5 cm in diameter) and the form of silver (either elemental Ag^0 or Ag^+ ions) that was released from the specimens needed to be determined. The actual amount of silver was quantified by dissolving the specimens in 5 mL of 69% nitric acid (HNO_3), followed by the addition of the releasing medium (acetate buffer, or SBF) to attain the 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 results were reported as average values from at least three measurements.

The release characteristic of silver from the nAg-containing gelatin fiber mats that had been exposed to the GTA vapor for 0.5 hr was assessed in acetate buffer or SBF as the releasing medium. The specimens cut from the fiber mat samples (circular disc; 1.5 cm in diameter) were immersed in 50 mL of the releasing medium at the skin or the physiological temperature of 32 or 37°C, respectively,

depending on the type of the releasing medium (i.e., 32°C for acetate buffer and 37°C for SBF). At a specified immersion period ranging between 0 and 7 d, the releasing medium was quantified for the amount of the released silver, using AAS. At each time point, the measurements were carried out in triplicate. The obtained data were carefully calculated to obtain the cumulative amount of the released silver. The cumulative release profiles of silver were expressed based on either the unit weight of the specimens or the unit weight of the actual amount of silver in the specimens.

5.3.6 Anti-bacterial evaluation

5.3.6.1 Zone of inhibition or Clear zone

The anti-bacterial activity of the e-spun fiber mats from the base gelatin solution and the 12 h-aged $AgNO_3$-containing gelatin solution after cross-linking with the moist GTA vapor for 0.5 h was tested against aerobic bacteria commonly found on burn wounds, i.e., *Escherichia coli* (Gram-negative; ATCC 25922), *Pseudomonas aeroginosa* (Gram-negative; ATCC 27853), *Staphylococcus aureus* (Gram-positive; ATCC 25023) and *Bacilllus subtilis* (Gram-positive; ATCC 6633). The assessment was conducted based on the disc diffusion method of the US Clinical and Laboratory Standards Institute (CLSI).

Both of the neat and the 0.75-2.00% $AgNO_3$-loaded gelatin fibers were cut into circular discs (1.5 cm in diameter) and sterilized with alcohol 70% with 30 min and then treated with sterilized distilled water before used and let the sample to dry in the air. Furthermore the vancomycin was used as antibacterial drug of *Staphylococcus aureus* and *Bacilllus subtilis.* Gentamicin was used as antibacterial drug of *Escherichia coli* and *Pseudomonas aeroginosa.* Each of the specimens and the drug were placed on the top of Difco™ Mueller Hinton Agar in a Petri dish and then incubated at 37 °C for 24 hr. Finally, the clear zone in agar plate was photographed to evaluate the antibacterial activity each specimens. If inhibitory concentrations are reached, there would be no growth of the microbes, which can be seen as a clear zone around the disc specimen.

5.3.6.2 AATCC Test Method 100 (Antibacterial Finishes on Textile Materials: Assessment of The American Association of Textile Chemists and Colorists) or Colonies count

This test method provides a quantitative procedure for evaluation of the degree of antibacterial activity. Bactericidal activity is intended or implied, quantitative evaluation is necessary. Quantitative evaluation also provided a clearer for possible uses of such treated textile materials.

Samples were cut in rectangular shape and sterilized with alcohol 70% with 30 min and then treated with sterilized distilled water before used and let the sample to dry in the air. The number of samples to be used is dependent on the fiber type and fabric construction. In this work, the samples in rectangular shape (~ 0.1 g or ~3.5×3.5 cm) of GT/$AgNO_3$ electrospun fibers mats will absorb the 1.0 ml of culture medium (10^5 CFU/ml), and leave no free liquid in the small plastic bag. Then these samples brought into incubator at 37°C for 24 hr. For control (neat GT), sample also cut in rectangular shape (0.1 g) of pure GT electrospun fibers mats will absorb the 1.0 ml of culture medium, and leave no free liquid in the small plastic bag and then brought these samples into incubator (37°C) for 24 hr. After 24 hr incubation, the bacteria were eluted from the samples. These samples transferred into the flask with screw cap containing 100 ml of sterilized distilled water and shake the flasks vigorously for 5 minutes, make serial dilutions with sterilized distilled water and spiral on plates (in triplicate) on nutrient agar. These palates brought into incubator at 37°C for 24 hr. Finally, the colonies on agar plate were photographed and count to evaluate the antibacterial activity. The number of bacteria present in this liquid (on agar plate) is determined, and the percentage reduction by the treated specimen was calculated.

Evaluation

Report bacterial counts as the number of bacteria per sample not as the number of bacteria per ml of neutralizing solution

Calculate percent reduction of bacteria by the specimen treatments by one of the following formulas:

$$100(B-A)/B = R$$

Where:

R = % reduction

A = the number of bacteria recovered from the incubated treated test specimen ($GT/AgNO_3$) after 37°C for 24 hr

B = the number of bacteria recovered from the incubated untreated control specimen (Neat GT) after incubation at 37°C for 24 hr

5.3.7 Indirect cytotoxicity evaluation

The indirect cytotoxicity evaluation of the 0.75-2.00% $AgNO_3$-loaded electrospun gelatin fiber mats after crosslinked with GTA vapor for 0.5 hr was conducted in adaptation from the ISO10993-5 standard test method in a 96-well tissue-culture polystyrene plate (TCPS; NunclonTM, Denmark) using normal human dermal fibroblasts (NHDF; twelfth passage) as reference. That of the neat gelatin fiber mats after crosslinked with GTA vapor for 0.5 hr was also conducted for comparison purpose. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen Corp., USA), supplemented by 10% fetal bovine serum (FBS; Invitrogen Corp., USA), 1% L-glutamine (Invitrogen Corp., USA) and 1% antibiotic and antimycotic formulation [containing penicillin G sodium, streptomycin sulfate, and amphotericin B (Invitrogen Corp., USA)].

The thickness of the fibers was 150 ± 20 μm. Both the neat gelatin fiber mats and 0.75-2.00% $AgNO_3$-loaded electrospun gelatin fiber mats specimens (circular discs; 15 mm in diameter) were pre-washed with 70% ethanol for 30 min, washed with autoclaved distilled water two times and left the sample to dry. The specimens were then immersed in serum-free medium (SFM; containing DMEM, 1% L-glutamine, 1% lactabumin, and 1% antibiotic and antimycotic formulation) for 24 hr in incubation to produce extraction media of varying concentration (i.e., 10, 5, and 0.5 $mg.mL^{-1}$). NHDF were separately cultured in wells of TCPS at 8,000 cells/well in serum-containing DMEM for 24 hr to allow cell attachment. The cells were then starved with SFM for 24 hr. After that, the medium was replaced with an extraction medium and cells were re-incubated for 24 hr. Finally, the viability of the cells cultured by each of the extraction media was determined with 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT) assay, with the viability of the cells cultured by fresh SFM being used as control.

The MTT assay is based on the reduction of the yellow tetrazolium salt to purple formazan crystals by dehydrogenase enzymes secreted from the mitochondria of metabolically-active cells. The amount of purple formazan crystals formed is proportional to the number of viable cells. First, each culture medium was aspirated and replaced with 25 μL/well of MTT solution at 5 mg□mL^{-1} for a 96-well TCPS. Secondly, the plate was incubated for 2 hr 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 the wavelength of 550 nm representing the viability of the cells was measured using a SpectraMax M2 microplate reader.

5.3.8 Cell attachment and morphological observation of cultured cells

In these experiments, both the neat gelatin fiber mats and 0.75-2.00% $AgNO_3$-loaded electrospun gelatin fiber mats specimens (circular discs; 1.5 cm in diameter) were pre-washed with 70% ethanol for 30 min, washed with autoclaved distilled water two times and left the sample to dry. Thereafter it was immersed with 500 μl of culture media (DMEM) for 24 hr into each well of the 24-well plate. For attachment study, $4.0x10^4$ of normal human dermal fibroblasts cells were seeded into each well of the culture plate and allowed to attach to the neat gelatin fiber mats and 0.75-2.00% $AgNO_3$-loaded electrospun gelatin fiber mats for 1 and 7 d. After 1 and 7 d, the culture medium was removed and then the cell-cultured electrospun fiber mats specimens were rinsed with PBS, the cells were then fixed with 500 μl/well of 3% glutaraldehyde solution (Electron Microscopy Science, USA). After 30 min, they were rinsed with PBS twice. After cell fixation, the specimens were dehydrated in an ethanol solution of varying concentration (i.e. 30, 50, 70, 90, and 100%, respectively) for about 2 min at each concentration. The specimens were then dried in 100% hexamethyldisilazane (HMDS; Sigma, USA) for 5 min and later let dry in air after removal of HMDS. After completely dried, the specimens will be mounted on an SEM stub, coated with gold, and observed by SEM.

5.3.9 Statistical Analysis

All the quantitative values were expressed as a mean ± standard deviation. Statistical comparisons were performed using one-way ANOVA with

SPSS 13.0 for Windows software (SPSS Inc., USA). *P* values <0.05 were considered statistically significant (n=3 for indirect cytotoxicity evaluation).

5.4 Results and Discussion

5.4.1 Formation of nAg in gelatin solutions and effect of aging time

The reduction of Ag^+ ions into elemental Ag^0 (i.e., nAg) in the $AgNO_3$-containing gelatin solutions at different aging times could be visualized from changes in the color of the solutions, i.e., from light yellow of the neat solution to brown of the 1 d-aged $AgNO_3$-containing solution to dark brown of the 10 d-aged $AgNO_3$-containing solution. Figure 5.1 shows UV-visible absorption spectra of both the base gelatin solution and the 0.75-2.00 wt.% $AgNO_3$-containing gelatin solutions that had been aged for various time intervals after preparation. No absorption of any kind was observed for the base gelatin solution. The surface plasmon absorption bands, centering around 420-430 nm, were first observed for the $AgNO_3$-containing gelatin solutions that had been aged for at least 12 h. Evidently, an increase in the aging time resulted in the observed increase in the intensity of the bands to gradually reach a plateau value at a long aging time (i.e., 7 d). This indicated that the amount of Ag^+ ions that was converted into nAg increased with an increase in the aging time and it is postulated that, at 7 d of aging, almost all of the Ag^+ ions were converted into nAg. Presumably from the relative constancy in the positions of the bands at different aging times, the average size of the nAg that were formed at different aging times was relatively the same. The possible mechanism of the silver nanoparticles formation was discussed in our previous [30].

Prior to e-spinning, the base gelatin and some of the 0.75-2.00% $AgNO_3$-containing gelatin solutions that had been 12 h-aged were measured for their shear viscosity and the conductivity and the results are summarized in Table 5.1. At a given aging time, the shear viscosity of the neat and the 0.75-2.00% $AgNO_3$-containing gelatin solutions was essentially similar, while the conductivity of the 0.75-2.00% $AgNO_3$-containing gelatin solution was greater than that of the base gelatin solution and increased with increasing the amount of $AgNO_3$ content, obviously a result of the presence of Ag^+ and NO_3^- ions in the solution.

5.4.2 Neat and nAg-loaded e-spun gelatin fiber mats

5.4.2.1 Morphology before and after cross-linking treatment

Table 5.2 shows selected SEM and TEM images of the e-spun fiber mats from both the base and the 12 h-aged 0.75-2.00% $AgNO_3$-containing gelatin solutions. Only cross-sectionally round fibers without the presence of beads were obtained. The diameters of the individual fibers obtained from these solutions were shown in Table 5.3. Electrospinning of the base and the 12 h-aged 0.75-2.00 wt.% $AgNO_3$-containing gelatin solutions resulted in the formation of smooth fibers, with the average diameters being 260.15 ± 45, 247.76 ± 38, 225.79 ± 35, 214.95 ± 39 and 206.25 ± 36 nm, respectively. Comparatively, Rujitanaroj [30] reported that the diameters of the neat and the 2.5% $AgNO_3$-loaded GT fibers ranged between 280 and 230 nm, respectively. Apparently, nAg were distributed throughout the fibers, the average diameters of these particles ranged between 10.00 and 13.00 nm. However, it should be emphasized that, at 12 h of aging, silver was present both as the Ag^+ ions and the elemental Ag^0, but, for convenience, nAg was used in a 'loose' term to denote both forms of silver that were present in the e-spun fiber.

Since gelatin is water-soluble, an e-spun gelatin fiber mat can easily dissolve either partially or completely to lose its fibrous structure when coming into contact with an aqueous medium or partially dissolve to lose its fibrous structure upon an exposure to a high humidity ambient, e.g., 80-90%, for a certain period of time [31]. To extend its use in applications that require an exposure to an aqueous medium or a high humidity, further cross-linking of the e-spun gelatin fiber mat is necessary. Among the various chemical systems used to cross-link an e-spun gelatin fiber mat (e.g., HDMI [33], EDC [34], and GTA vapor [31,32] GTA is seemingly the most suitable one, as it is economical and does not compromise the fibrous structure of the e-spun membrane. Here, the neat and the nAg-containing e-spun gelatin fiber mats were further cross-linked by saturated vapor from 50 vol.% GTA aqueous solution for either 0.5 h, followed by a heat treatment at 110°C for 24 h. Cross-linking treatment in this matter did not affect the amount of nAg in the cross-linked e-spun fiber mats.

Table 5.2 shows selected SEM images of the e-spun fiber mats from both the neat and the 12 h-aged 0.75-2.00% $AgNO_3$-containing gelatin solutions after having been cross-linked with the moist GTA vapor for either 0.5 h. Evidently, exposing the fiber mats in the chamber caused some fibers to fuse to one another at touching points, a result of the partial dissolution of the fiber segments when they came into contact with the moisture-rich GTA vapor. In addition, both the neat and the nAg-containing e-spun gelatin fiber mats, after cross-linking, changed their color from white to brown (for the fiber mats that had been cross-linked for 0.5 h) and slightly shrunk from their original dimensions. The change in color of gelatin upon cross-linking with GTA is caused by the formation of aldimine linkages (-CH=N-) between the free amino groups of lysine or hydroxylysine amino acid residues of the protein and the aldehyde groups of GTA [35,36]. Moreover, the shrinkage of the fiber mats is responsible for the observed decrease in the size of inter-fibrous pores (see Table 5.2) as well as the observed decrease in the thickness of the fiber mats and the observed increase in the diameters of the individual fibers (see Table 5.3).

5.4.2.2 Weight loss and water retention behavior

To assess the degree of cross-linking, both the neat and the nAg-containing e-spun gelatin fiber mats that had been cross-linked with the moist GTA vapor for either 0.5 h were investigated for weight loss and water retention behavior after submersion in acetate buffer, or SBF for various submersion time intervals (i.e., 1, 3, 5, and 7 d). The results of such analyses are available as shown in Figure 5.2 and Figure 5.3. For a given exposure time in the cross-linking chamber and a given type of medium, the weight loss and the water retention ability for both types of the e-spun gelatin fiber mats increased with an increase in the submersion time. At any given submersion time, both the weight loss and the water retention of the fiber mat samples was the greatest when they were submerged in the acetate buffer. Though not shown, the physical integrity of the neat and the 0.75-2.00% $AgNO_3$-loaded e-spun GT fiber mats were retained after submersion in the medium at 32 °C and 37 °C for 7 d.

5.4.2.3 Mechanical integrity

In the wound dressing material containing Ag nanoparticles, the Ag will be released in the aqueous state, so for the mechanical properties measurement, all of specimens were submerged in acetate buffered solution (at the skin temperature of 32°C, pH 5.5) for 1 day to obtain swelling specimens. The results on the mechanical assessment of both the neat and the nAg-containing e-spun gelatin fiber mats that had been cross-linked for 0.5 h are presented in Table 5.4. Before submersion in acetate buffer, both the neat and the 0.75-2.00% $AgNO_3$-loaded e-spun GT fibers mats have comparable valued of strain at maximum load (%), stress at maximum load (MPa), strain at break (%) and tensile strength (MPa) with 8.00-10.00%, 14.50-17.50 MPa, 8.00-10.0% and 4.00-6.00 MPa, respectively. On the other hands, both the strain at break and the strain at maximum load of the neat gelatin fiber mat and 0.75-2.00% $AgNO_3$-loaded gelatin fiber mat samples were increased after submerged in acetate buffer from 8.00-10.0% and 8.00-10.00% to 45-65% and 45-66%, respectively due to water retention behavior and elasticity of e-spun at wet state. Tensile strength and stress at maximum load of the neat gelatin fiber mat and $AgNO_3$-loaded gelatin fiber mat samples were decreased after immersion in acetate buffer from 4.00-6.00 MPa and 14.50-17.50 MPa to 0.36-0.45 MPa and 1.18-1.68 MPa due to the weight loss behaviors and the release characteristic of silver from the nAg-containing e-spun gelatin fiber mats.

5.4.2.4 Release characteristic of silver

Prior to investigating the release characteristic of silver from the nAg-containing e-spun gelatin fiber mats that had been cross-linked for 0.5 h, the actual amount and the form of silver that was released from the materials needed to be identified. To determine the amount of silver in these samples, the uncross-linked fiber mat specimens which had been prepared from the gelatin solution containing 0.75-2.00 wt.% $AgNO_3$ were first dissolved in 5 mL of 69% HNO_3, followed by the addition of an appropriate releasing medium (i.e., acetate buffer, or SBF) to attain the final volume of 50 mL. The obtained silver-containing solutions in such media were then determined for the actual contents of the as-loaded silver by means of AAS as shown in Table 5.5.

These nAg-containing fibrous materials were prepared from the gelatin solution containing 0.75-2.00% wt.% $AgNO_3$ that had been aged for 12 h prior to e-spinning. At 12 h of aging, silver was present both in the ionic and the metallic nanoparticle forms, as previously discussed. Some of these ions were reduced into elemental Ag^0 upon aging. Evidently, the amounts of both the remnant Ag^+ ions (the ones that remained in the ionic form) and the Ag^+ ions that resulted from the dissolution of the as-formed elemental Ag^0 constituted to ca. 94-98% (acetate buffer) and 83-87% (SBF) of the initial amount of Ag^+ loaded in the gelatin solution. As proven by the visual observation of the sample solution containing silver that was released from the 0.5 h-cross-linked nAg-containing e-spun gelatin fiber mat specimen that had been submerged in acetate buffer or SBF for 7 d, only silver in the ionic form was released into the sample solution. Pal (2002) [37] showed that, in an aqueous medium containing a nucleophile (e.g., $NaBH_4$, SCH^-, and I^-), the dissolution of silver is possible due to the significant decrease in the reduction potential and the redox reaction for silver dissolution can be written as

$$4Ag + O_2 + 2H_2O \leftrightarrow 4Ag^+ + 4OH^- \quad (3)$$

Here, it is postulated that the as-formed elemental Ag^0 dissolved readily upon the contact with the releasing medium and both the remnant and the dissolved Ag^+ ions were released into the medium during the release studies.

The release characteristic of Ag^+ ions from 0.5 h-cross-linked nAg-containing e-spun gelatin fiber mat samples was investigated by the total immersion method in one of the releasing media (i.e., the acetate buffer, and SBF). The cumulative amount of Ag^+ ions released from these materials is reported as the weight of Ag^+ ions released (in ppm) divided by the weight of the specimens (in g) in Figure 5.4. Evidently, the cumulative amount of Ag^+ ions released from these samples in the acetate buffer and SBF occurred rather rapidly during the first 60 min after submersion in the releasing medium and it increased gradually afterwards. As expected, the maximum amount of Ag^+ ions released from these materials increased with increasing the initial amount of $AgNO_3$ loaded in the spinning solutions.

Alternatively, the cumulative amount of Ag^+ ions released from these materials can also be reported as the concentration of the as-released Ag^+

ions in the releasing media (i.e., in ppm or mg/L of the releasing media) divided by the actual weight of the specimens (in g) and as the percentage of the weight of the as-released Ag^+ ions divided by the actual weight of Ag^+ ions in the specimens (see Figure 5.5). The final cumulative release of Ag+ ions can be concluded in Table 5.6.

5.4.2.5 Antibacterial activity

The potential for use of the nAg-containing e-spun gelatin fiber mats as functional wound dressings was assessed by observing their anti-bacterial activity against some common bacteria found on burn wounds, i.e., *E. coli*, *P. aeroginosa*, *S. aureus* and *B. subtilis* based on the disc diffusion method (in Agar and Blood agar) and AATCC Test Method 100 or Colonies count. The activity of the neat e-spun gelatin fiber mats against these bacteria was used as control.

Zone of inhibition or clear zone tested in Agar and Blood agar plate as shown in Table 5.7 and Table 5.8, respectively. According to these results obtained, neat e-spun gelatin fiber mat specimens showed no activity against the tested bacteria. For the nAg-containing specimens, inhibitory zones were evident. The zone of inhibition or clear zone increased with increasing the initial amount of $AgNO_3$ loaded in the spinning solutions. Specifically, the antibacterial activity of the nAg-containing specimens, regardless of the sample types, was the greatest against *B.subtilis*, followed by *S. aureus*, *P. aeroginosa*, and, *E. coli* respectively (see Figure 5.6). Relatively, the antibacterial effeiciency against *P. aeroginosa* and *E. coli* are lower than that against *S. aureus*, probably because of the difference in cell wall between gram-positive and gram-negative bacteria. The cell wall of *P. aeroginosa* and *E. coli*, which consists of lipids, proteins and lipopolysaccharides (LPS), provide effective protection against biocides. However, the cell wall of gram-negative bacteria, such as *S. aureus*, does not consist of LPS [38].

AATCC Test Method 100 (Antibacterial Finishes on Textile Materials: Assessment of The American Association of Textile Chemists and Colorists) or Colonies count provides a quantitative procedure for evaluation of the degree of antibacterial activity. Bactericidal activity is intended or implied, quantitative evaluation is necessary. Quantitative evaluation also provided a clearer for possible uses of such treated textile materials. According to these results obtained, the neat e-spun gelatin fiber mat specimens showed no activity against the

tested bacteria while the nAg-containing specimens showed the antibacterial activity against these bacteria. The colonies on agar plate were photographed as shown in Table 5.9 and 5.10. Quantitative evaluation with antibacterial activity of the 0.75-2.00% $AgNO_3$-loaded e-spun GT fibers mats against *Staphylococus aureus, Bacillus subtilis, Escherichia coli*, and *Pseudomonas aeruginosa* as shown in Table 5.11. From these results showed that $AgNO_3$-loaded electrospun gelatin fibers mats show high efficiency antibacterial capacities increase with increasing the silver content because the percentage of reduction reached 99.99 % with all of bacteria, indicating that the silver particles are responsible for the antibacterial activity of the $AgNO_3$-loaded electrospun gelatin fibers mats and this activity is quite strong.

5.4.2.6 Indirect cytotoxicity evaluation

The potential for use of the 0.75-2.00% $AgNO_3$-loaded e-spun GT fiber mats as wound dressings was assessed by evaluating the cytotoxicity of these materials, using the neat GT fiber mats as internal control. Even though it is known that gelatin is biocompatible with cells. However, the as-spun gelatin fiber mats were crosslinked with GTA, known toxic organic substances. The viability of the normal human dermal fibroblasts (NHDF) that had been cultured with the extraction media from these materials in comparison with that of the cells that had been cultured with fresh culture medium (i.e., control) is illustrated in Figure 5.7. Three extraction ratios of the extraction media (i.e., 10, 5, and 0.5 mg.mL-1) were investigated for 1 day. Apparently, the neat GT fiber mats were non-toxic to the cells, as the viability of the cells that had been cultured with extraction media from these materials at all extraction ratios investigated ranged between ~87 and ~97% (relative to the viability of the cells that had been cultured with fresh culture medium). At the medium extraction ratio investigated (i.e., 5 mg.mL-1), only the 0.75-1.00% $AgNO_3$-loaded materials appeared to be non-toxic to the cells, with the viability of the cells ranging between ~90 and ~86%, respectively. While 1.50-2.00% $AgNO_3$-loaded materials were toxic to the cells. At greater extraction ratios investigated (i.e., 10 mg.mL-1), all of the $AgNO_3$-loaded materials were toxic to the cells, as the viability of the cells was lower than 80% (i.e., ranging between ~46 and ~67%). It is assumed that the toxicity of the $AgNO_3$-loaded materials should be a result of highly amount of Ag^+ or NO_3^- presented in the extraction media.

For the 5 mg.mL-1 of extraction ratio will be investigated for 7 d of the indirect cytotoxicity evaluation. Apparently, the neat GT fiber mats were non-toxic to the cells, with the viability of the cells was ~91% and the 0.75-1.00% $AgNO_3$-loaded materials appeared to be non-toxic to the cells, with the viability of the cells ranging between ~88 and ~86%, respectively. While 1.50-2.00% $AgNO_3$-loaded materials were toxic to the cells. The obtained results revealed that the 0.75-1.00% $AgNO_3$-loaded electrospun GT fibers mats were non-toxic to the normal human dermal fibroblasts (NHDF).

5.4.2.7 Cell attachment and Morphological observation of cultured cells

Attachments of cells are the important aspects of wound dressing materials. In this work, the 0.75-1.00% $AgNO_3$-loaded electrospun gelatin fibers mats were evaluated in comparison with neat gelatin fibers mats and TCPS. To evaluate cellular behavior on TCPS (i.e. controls), and electrospun fibers mats, Normal human dermal fibroblasts (NHDF) were seeded and cultured on all substrates. SEM observations were carried out in order to observe cell morphology and interaction between cells and cells and the electrospun fibers mats. SEM images of NHDF that were cultured on the 0.75-1.00% $AgNO_3$-loaded electrospun gelatin fibers mats, neat gelatin fibers mats and a glass substrates (i.e. controls) at a different time in culture were shown in Table 5.12 and 5.13. For the neat gelatin fibers mats, these SEM images confirmed that cells were cultured on the electrospun fibers mats exhibited the expanded shape with discrete branches to help attach themselves on the fiber surfaces after 1 day and at longer culturing times, 7 days, the cells on the electrospun fibers mats expanded even more and could cover on the whole area of electrospun fibers mats very well. In contrast, the 0.75-1.00% $AgNO_3$-loaded electrospun gelatin fibers mats, the cells remained the round shape (1 day) and expanded even more at longer time in culture (7 days). Normally, the cells should expanded shape with discrete branches to help attach them on the fiber surface with 1 day. It showed that silver has significant killing or inhibitory effect on the cell growth of the fibroblast. Silver is toxic to both keratinocytes and fibroblasts in monolayer culture [39, 40]. These results suggest that silver-based dressings

should be used with caution in situations where rapidly proliferating cells may be harmed as in donor sites, superficial burns, and application of cultured cells.

5.5 Conclusion

Silver ions (Ag^+ ions), known for their broad-spectrum antimicrobial activity, were reduced into silver nanoparticles (nAg) in a base gelatin (type A, porcine skin, 170-190 Bloom) solution (22% w/v in 70 vol% acetic acid) containing 0.75-2.00 wt% $AgNO_3$ (based on the weight of the gelatin powder). The presence of nAg in the $AgNO_3$-containing gelatin solution was first realized after it had been aged for at least 12 h. The amount of nAg formed increased monotonically with increasing aging time. Both the base and the 12 h-aged 0.75-2.00 wt.% $AgNO_3$-containing gelatin solutions were fabricated into ultrafine fibers by electrospinning (15 kV/20 cm). The average diameters of these fibers were 260, 248, 226, 215 and 206 nm, respectively. The average diameters of the as-formed nAg ranged between 10.18 and 12.80 nm. Both the neat and the nAg-containing gelatin fiber mats were further cross-linked, to improve their stability in an aqueous medium or a high humidity atmosphere, by saturated vapor from 50 vol% glutaraldehyde (GTA) aqueous solution for 0.5 h, followed by a heat treatment at 110°C for 24 h. Cross-linking not only caused the color of the cross-linked gelatin fibers to change, but also was responsible for the fusing and shrinking of the cross-linked fiber mats. The stress at maximum load for all of the obtained fiber mats ranged between 14.50 and 17.50 MPa, while the strain at maximum load ranged between 8.00 and 10.00%%. Weight loss and water retention of both the neat and the nAg-containing gelatin fiber mats in acetate buffer (pH 5.5), and simulated body fluid (SBF; pH 7.4) were found to increase with increasing submersion.

The release characteristic of Ag^+ ions from 0.5 h-cross-linked nAg-containing e-spun gelatin fiber mats was investigated by the total immersion method in acetate buffer (at the skin temperature of 32°C), and SBF (at the physiological temperature of 37°C). The cumulative release of Ag^+ ions from the samples in the acetate buffer occurred rather rapidly during the first 60 min after submersion in the

releasing medium, and increased gradually afterwards; while those in SBF less than that of in acetate buffer during the first 60 min. The antibacterial activity of these materials was greatest against *Bacillus subtilis*, followed by *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli*, respectively. For quantitative evaluation with antibacterial activity of the 0.75-2.00% $AgNO_3$-loaded e-spun GT fibers mats, it is found that the percentage of reduction reached 99 % with all of bacteria, indicating that the silver particles are responsible for the antibacterial activity of the $AgNO_3$-loaded electrospun gelatin fibers mats and this activity is quite strong. Finally, the potential for use of the 0.75-1.00% $AgNO_3$-loaded e-spun GT fiber mats as wound dressings was assessed by investigating the cytotoxicity of these materials against normal human dermal fibroblasts. The results showed that only the extraction media from the 0.75-1.00% $AgNO_3$-loaded e-spun GT fiber mats at the extraction ratios of 10 $mg \cdot mL^{-1}$ were toxic to the cells for 1 d.

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Table 5.1 Shear viscosity and conductivity of the base gelatin (GT) and some of the 0.75-2.00% $AgNO_3$-containing GT solutions that had been 12 h-aged after preparation

Type of GT solution	**Shear viscosity (mPa.s)**	**Electrical conductivity (□S.cm^{-1})**
Neat	435 ± 1.1	1257.67 ± 1.53
With 0.75% $AgNO_3$	442 ± 1.0	1306.67 ± 3.21
With 1.00% $AgNO_3$	441 ± 0.5	1330.33 ± 0.58
With 1.50% $AgNO_3$	445 ± 1.5	1349.67 ± 1.53
With 2.00% $AgNO_3$	448 ± 1.0	1355.30 ± 1.00

Table 5.2 Selected SEM and TEM images of fibers before and after cross-linked 0.5 h and the particles size of Ag nanaparticles

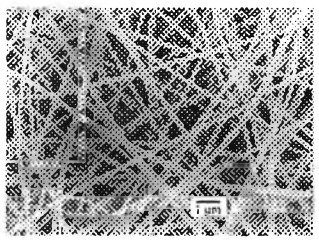
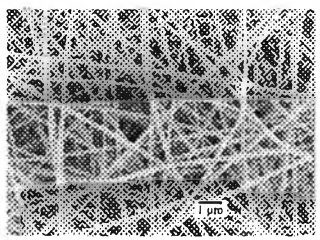
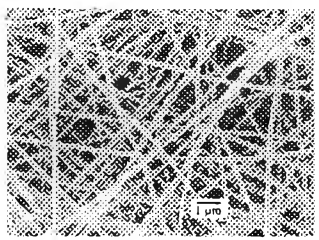
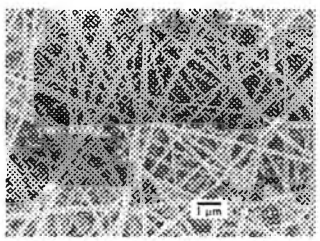
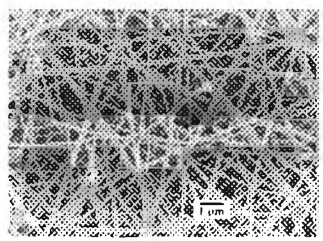
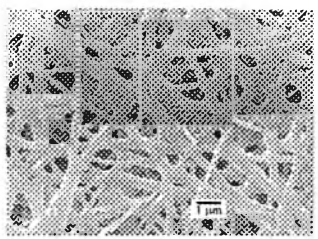
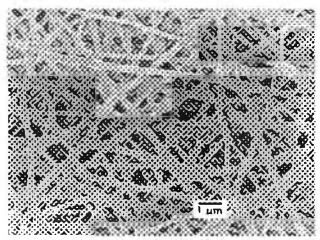
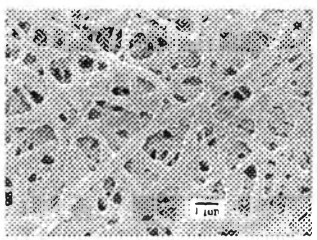
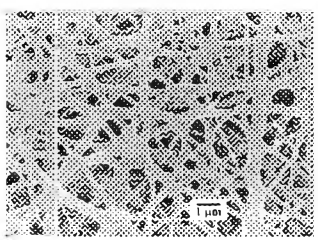
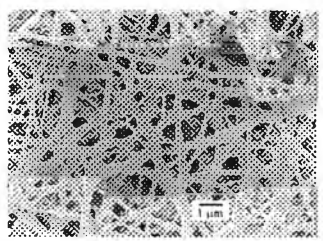
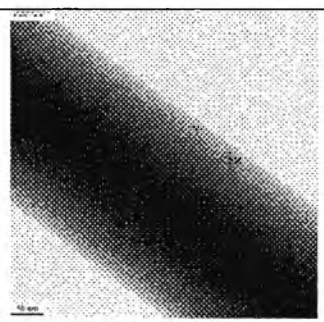
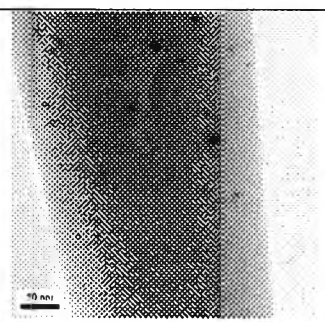
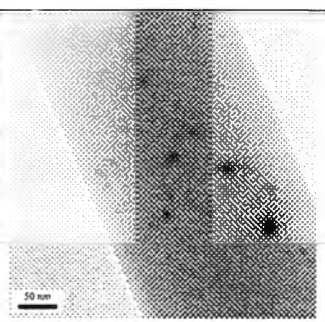
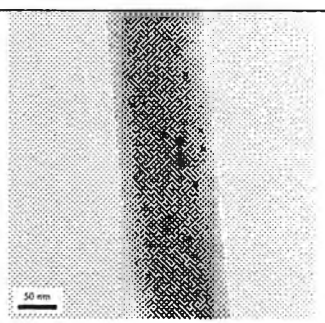
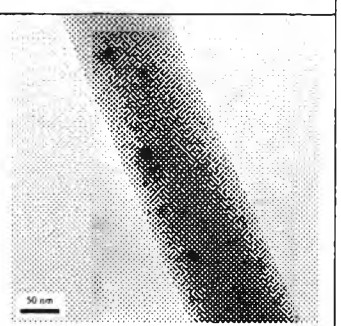
Types of material	Neat gelatin fibers mats	0.75% $AgNO_3$-loaded e-spun gelatin fiber mats	1.00% $AgNO_3$-loaded e-spun gelatin fiber mats	1.50% $AgNO_3$-loaded e-spun gelatin fiber mats	2.00% $AgNO_3$-loaded e-spun gelatin fiber mats
Fibers before cross-linked 0.5 h					
Fibers after cross-linked 0.5 h					
Particles size of Ag nanaparticles in e-spun GT fiber		 11.68 ± 3.5 nm	 12.80 ± 3.9 nm	 10.10 ± 3.5 nm	 10.18 ± 2.8 nm

Table 5.3 The average diameter and thickness of e-spun fibers mats

Types of material	Before cross-linked 0.5 h		After cross-linked 0.5 h	
	The average diameter of the as-spun (nm)	**Thickness of the as-spun (μm)**	**The average diameter of the as-spun (nm)**	**Thickness of the as-spun (μm)**
Neat gelatin fibers mats	260.15 ± 44.75	212.29 ± 33.46	392.99 ± 68.95	125.75 ± 31.14
0.75% $AgNO_3$-loaded e-spun gelatin fiber mats	247.76 ± 38.17	200.13 ± 29.80	385.64 ± 75.08	140.25 ± 12.37
1.00% $AgNO_3$-loaded e-spun gelatin fiber mats	225.79 ± 34.69	185.86 ± 15.42	373.59 ± 58.93	137.50 ± 17.83
1.50% $AgNO_3$-loaded e-spun gelatin fiber mats	214.95 ± 38.62	185.63 ± 29.56	362.05 ± 74.68	142.13 ± 26.16
2.00% $AgNO_3$-loaded e-spun gelatin fiber mats	206.25 ± 36.35	183.63 ± 29.40	349.32 ± 72.14	134.87 ± 10.60

Table 5.4 The mechanical assessment of both the neat and the nanoAg^0-containing e-spun gelatin fiber mats that had been cross-linked for 0.5 h before and after immersion in acetate buffer for 1 day

Types of Materials	Strain at Maximum Load (%)		Stress at Maximum Load (MPa)		Strain at Break (%)		Tensile strength (MPa)	
	Before immersion	After immersion	Before immersion	After immersion	Before immersion	After immersion	Before immersion	After immersion
Neat gelatin fibers mats	9.65 ± 3.04	55.00 ± 8.65	15.27 ± 2.68	1.42 ± 0.40	9.72 ± 3.10	53.79 ± 9.85	4.58 ± 0.80	0.45 ± 0.12
0.75% $AgNO_3$-loaded e-spun gelatin fiber mats	8.05 ± 1.09	58.81 ± 5.61	14.87 ± 2.38	1.20 ± 0.23	8.32 ± 2.51	60.53 ± 14.50	4.18 ± 1.05	0.40± 0.15
1.00% $AgNO_3$-loaded e-spun gelatin fiber mats	8.59 ± 2.42	50.36 ± 6.64	17.30 ± 3.49	1.29 ± 0.15	8.75 ± 1.54	52.06 ± 14.70	5.19 ± 1.09	0.35 ± 0.12
1.50% $AgNO_3$-loaded e-spun gelatin fiber mats	9.33 ± 1.68	55.35 ± 8.61	16.58 ± 2.24	1.68 ± 0.53	9.35 ± 3.45	56.23 ± 10.11	4.98 ± 1.04	0.42 ± 0.12
2.00% $AgNO_3$-loaded e-spun gelatin fiber mats	9.28 ± 1.33	48.64 ± 9.51	17.18 ± 4.54	1.18 ± 0.34	9.68 ± 2.99	50.15 ± 11.91	5.70 ± 1.08	0.36 ± 0.13

Table 5.5 Actual amount of silver incorporated in $AgNO_3$-loaded electrospun GT fiber mats

Initial amount of $AgNO_3$ in spinning solution (wt.%)	**Actual amount of silver based on the initial amount of the $AgNO_3$ loaded (%)**	
	$AgNO_3$-loaded electrospun GT fiber mats in acetate buffer	**$AgNO_3$-loaded electrospun GT fiber mats in SBF**
0.75	96.5 ± 0.4	84.5 ± 0.2
1.00	93.6 ± 0.1	82.8 ± 0.1
1.50	97.5 ± 0.1	84.9 ± 0.3
2.00	95.6 ± 0.1	86.5 ± 0.3

Table 5.6 Cumulative release of Ag^+ ions in the concentration of Ag^+ ions released into the medium (ppm) divided by the actual weight of specimens (in g) and the percentage of the weight of Ag^+ ions released divided by the actual weight of Ag^+

Releasing medium	Cumulative release of Ag^+ ions (ppm/g)	Cumulative release of Ag^+ ions (percentage of the weight of Ag^+ ions released / the actual weight of $Ag^{+)}$
Acetate buffer	91.74 ± 0.27	90.94 ± 0.23
	118.79 ± 0.26	91.36 ± 0.18
	179.84 ± 0.37	98.78 ± 0.20
	237.60 ± 0.64	98.36 ± 0.27
SBF	60.68 ± 0.1	85.57 ± 0.14
	95.99 ± 0.13	90.88 ± 0.12
	148.82 ± 0.23	93.29 ± 0.15
	209.91 ± 0.28	98.01 ± 0.13

Table 5.7 Antibacterial activities of the neat GT fibers mat and the 0.75-2.00% $AgNO_3$-loaded e-spun GT fibers mat against *Staphylococcus aureus, Bacillus subtilis, Escherichia coli* and *Pseudomonas aeruginosa* in Agar plates

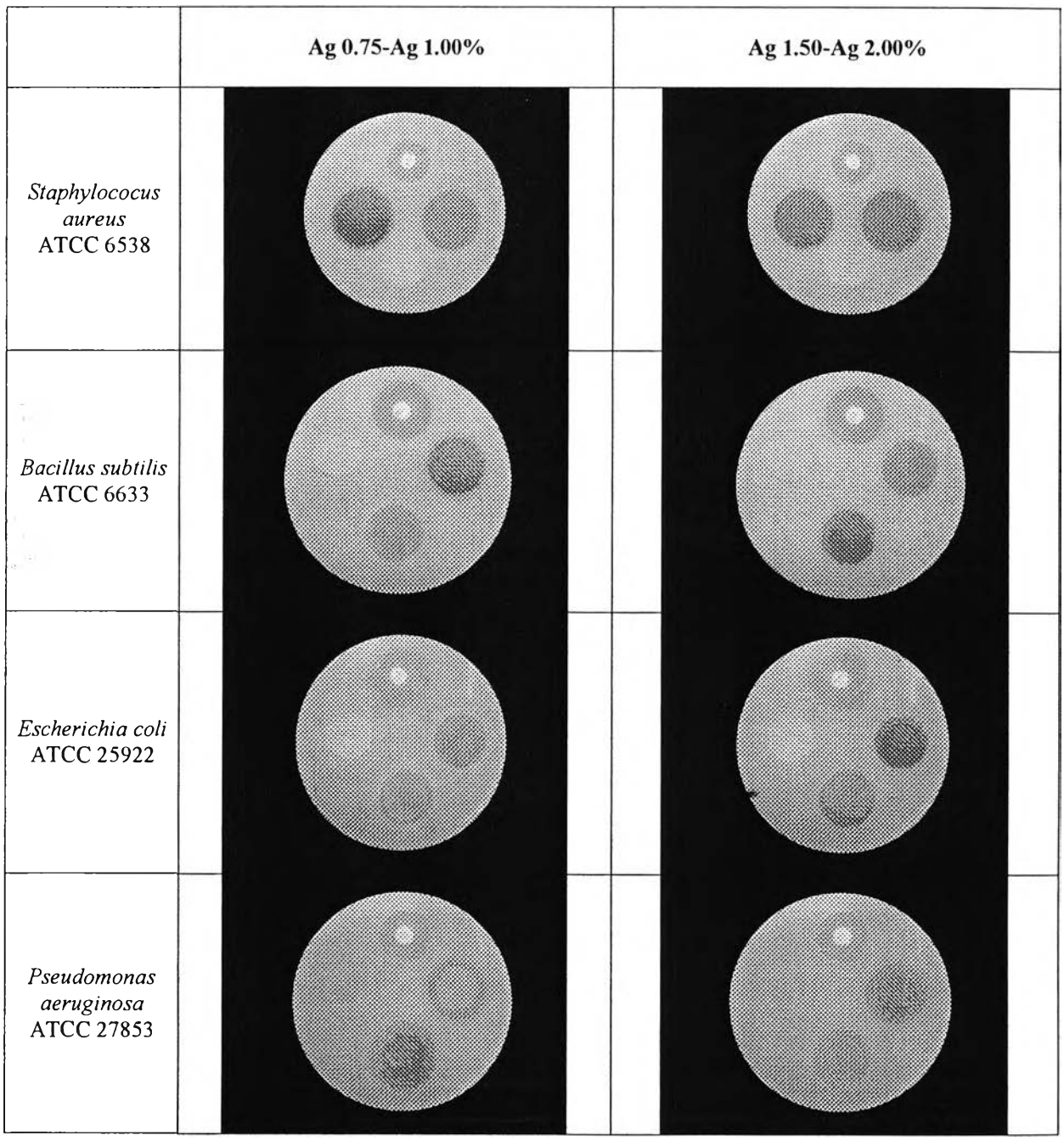


Table 5.8 Antibacterial activities of the neat GT fibers mat and the 0.75-2.00% $AgNO_3$-loaded e-spun GT fibers mat against *Staphylococus aureus, Bacillus subtilis, Escherichia coli*, and *Pseudomonas aeruginosa* in Blood Agar plates

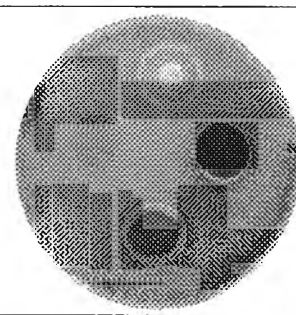
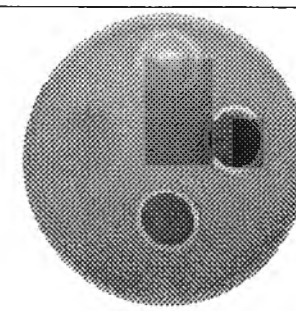
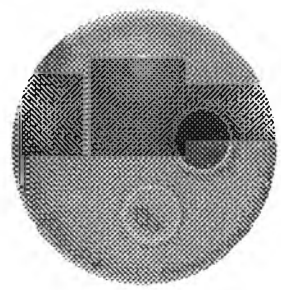
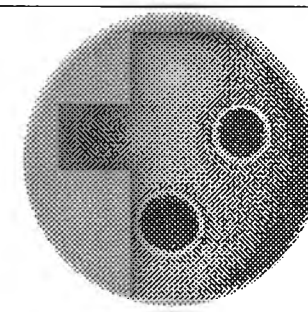
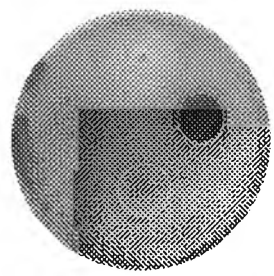
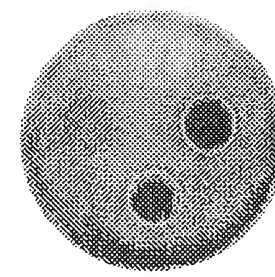
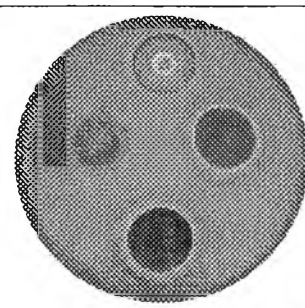
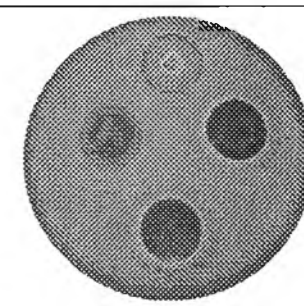
	Ag 0.75-Ag 1.00%	**Ag 1.50-Ag 2.00%**
Staphylococus aureus ATCC 6538		
Bacillus subtilis ATCC 6633		
Escherichia coli ATCC 25922		
Pseudomonas aeruginosa ATCC 27853		

Table 5.9 Antibacterial activities of the neat GT fibers mat and the 0.75-2.00% $AgNO_3$-loaded e-spun GT fibers mat against *Staphylococus aureus* and *Bacillus subtilis* in AATCC Test Method 100

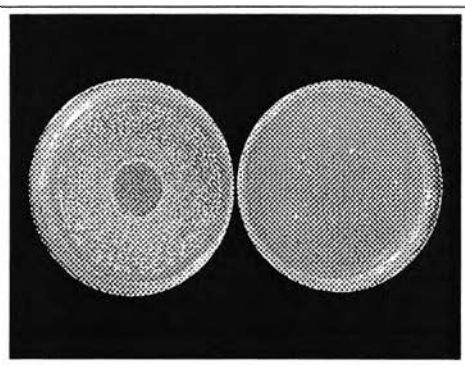
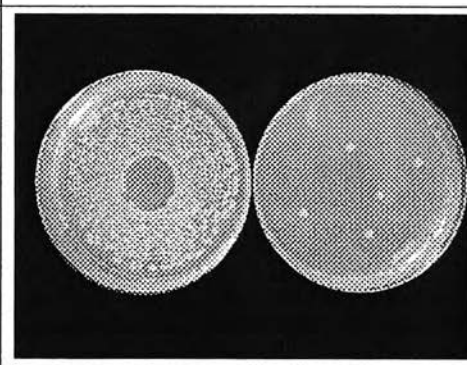
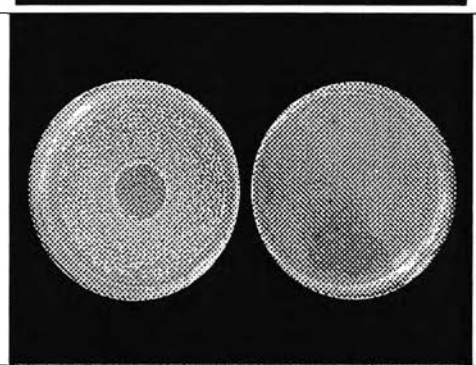
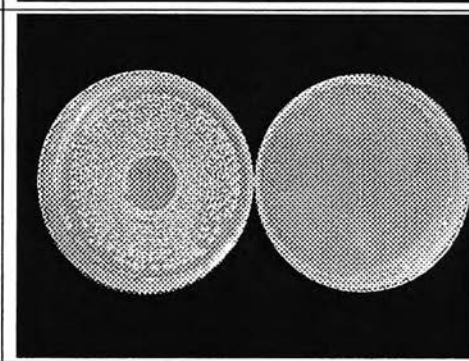
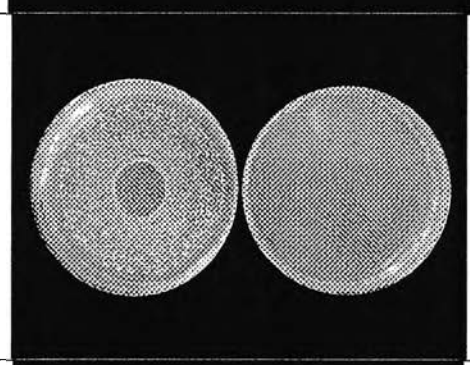
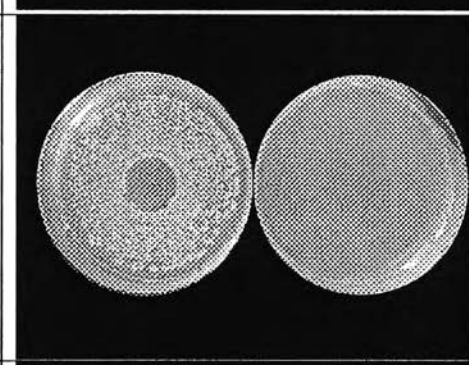
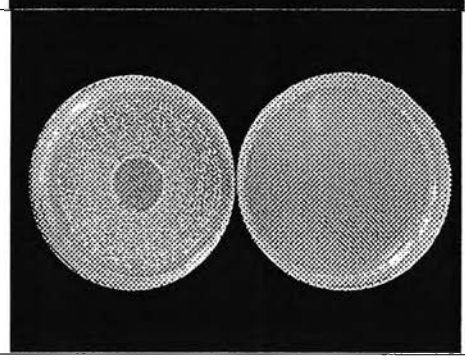
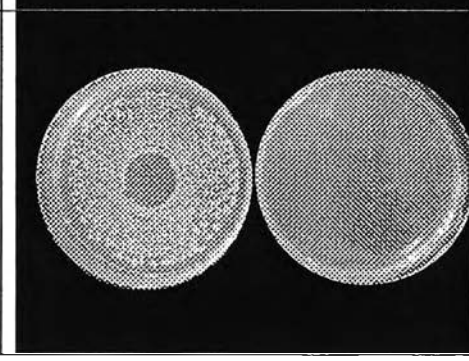
Materials	***Staphylococus aureus*** ATCC 6538	***Bacillus subtilis*** ATCC 6633
Ag 0.75%		
Ag 1.00%		
Ag 1.50%		
Ag 2.00%		

Table 5.10 Antibacterial activities of the neat GT fibers mat and the 0.75-2.00% $AgNO_3$-loaded e-spun GT fibers mat against *Escherichia coli* and *Pseudomonas aeruginosa* in AATCC Test Method 100

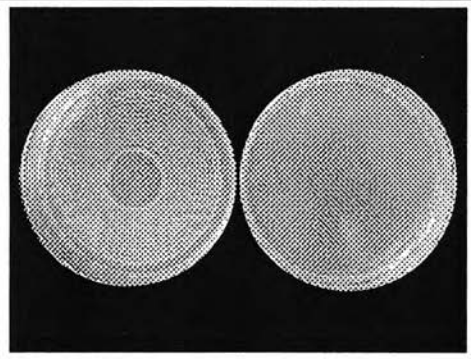
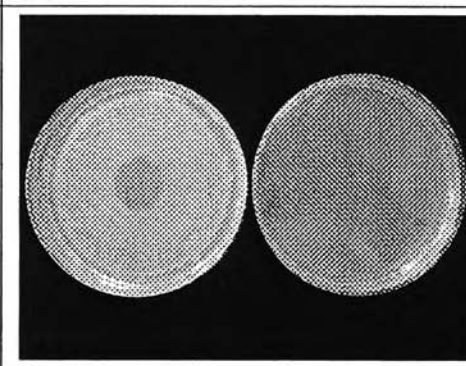
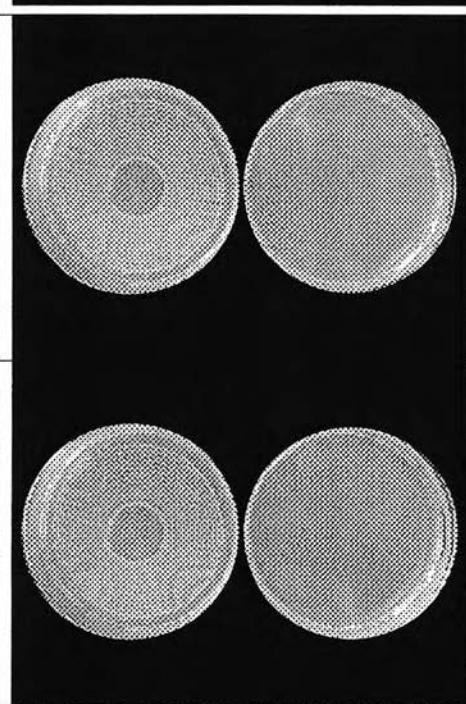
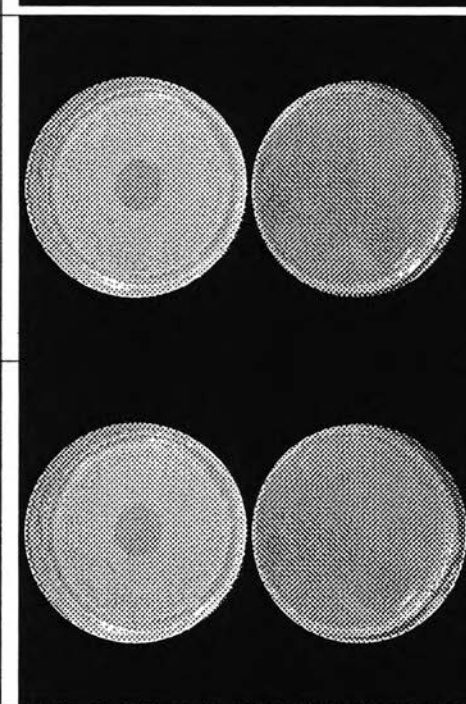
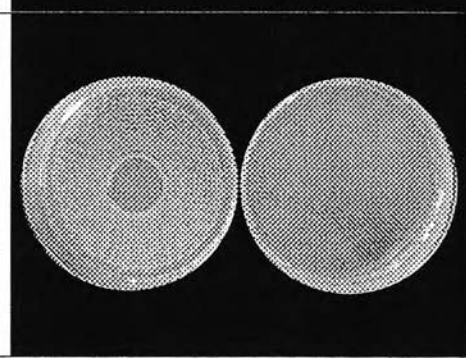
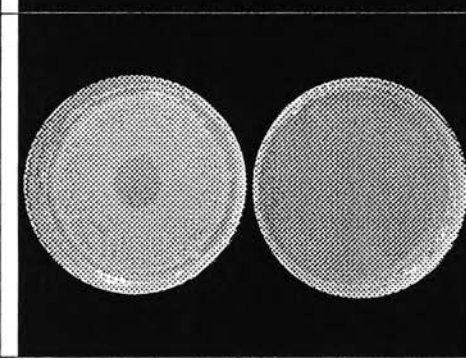
Materials	***Escherichia coli*** ATCC 25922	***Pseudomonas aeruginosa*** ATCC 27853
Ag 0.75%		
Ag 1.00%		
Ag 1.50%		
Ag 2.00%		

Table 5.11 Quantitative evaluation with antibacterial activity of the 0.75-2.00% $AgNO_3$-loaded e-spun GT fibers mats against *Staphylococus aureus*, *Bacillus subtilis*, *Escherichia coli*, and *Pseudomonas aeruginosa*

Materials	***Staphylococus aureus***	***Bacillus subtilis***	***Escherichia coli***	***Pseudomonas aeruginosa***
0.75% $AgNO_3$ loaded e-spun GT fibers mats	99.83%	99.99%	99.80%	99.80%
1.00% $AgNO_3$ loaded e-spun GT fibers mats	99.99%	99.99%	99.99%	99.99%
1.50% $AgNO_3$ loaded e-spun GT fibers mats	99.99%	99.99%	99.99%	99.99%
2.00% $AgNO_3$ loaded e-spun GT fibers mats	99.99%	99.99%	99.99%	99.99%

Table 5.12 Selected SEM micrographs of NHDF cells proliferated on the neat gelatin fibers mats and the 0.75-1.00% $AgNO_3$-loaded electrospun gelatin fibers mats at 1 day of cell culture; Mag = 500x and 3500x

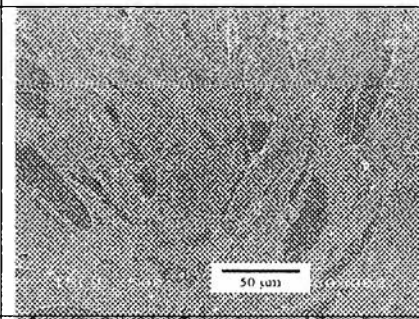
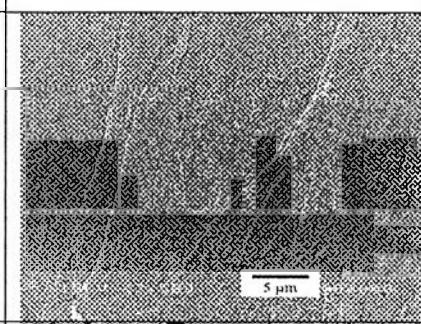
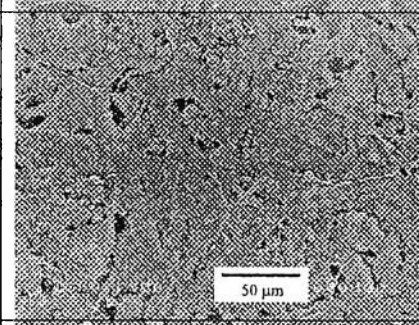
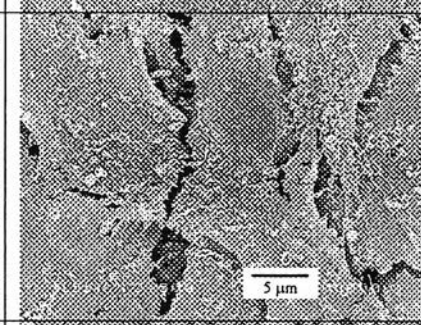
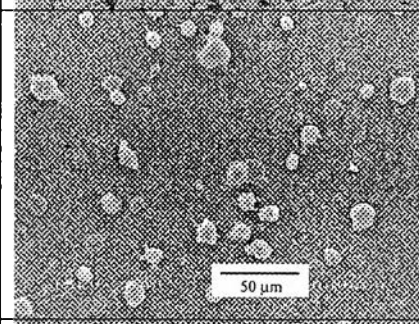
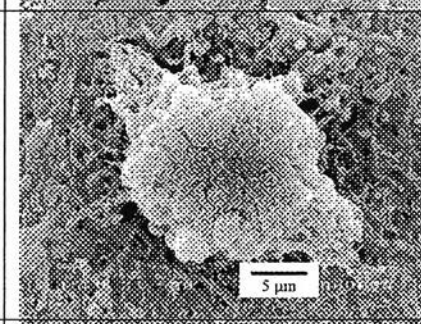
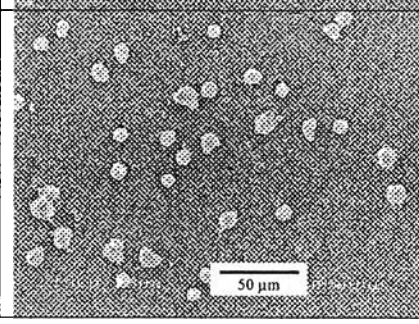
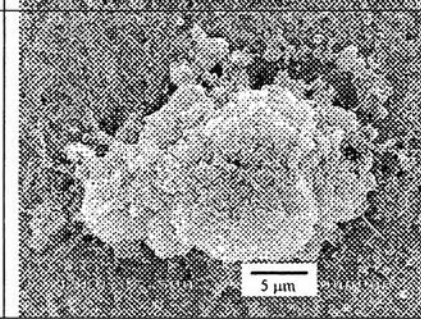
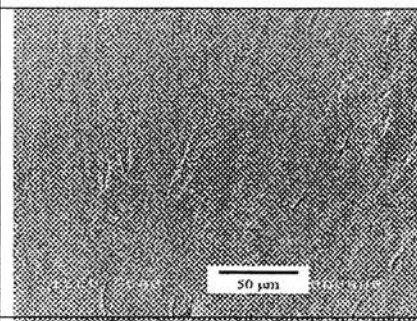
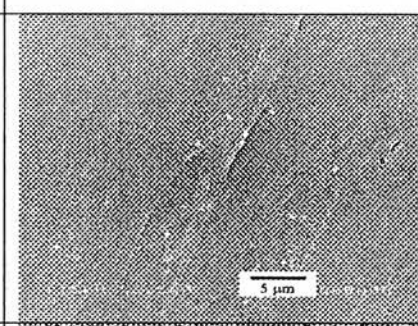
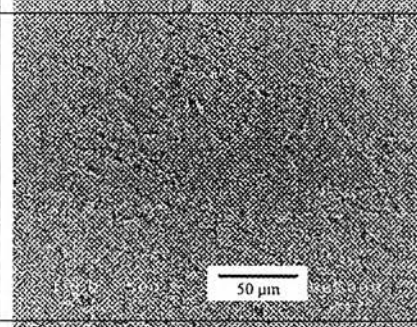
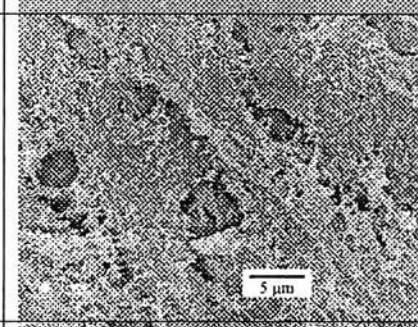
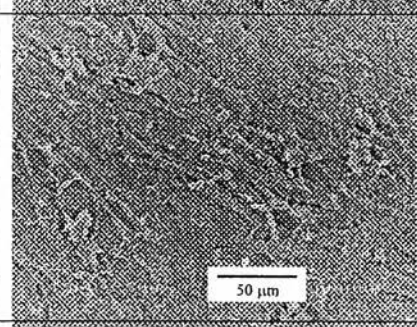
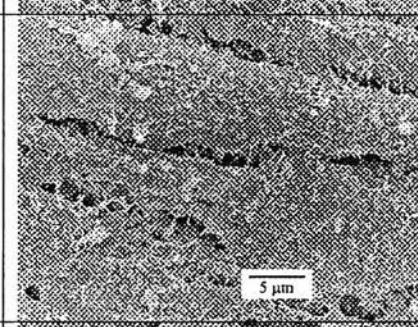
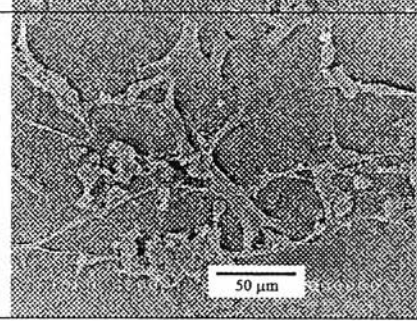
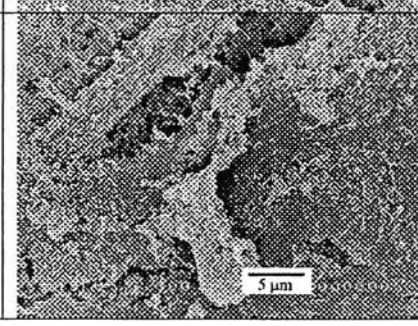
Materials	500x	3500x
Polystyrene plate		
E-spun GT		
$AgNO_3$ 0.75 %-loaded e-spun GT fiber mats		
$AgNO_3$ 1.00%-loaded e-spun GT fiber mats		

Table 5.13 Selected SEM micrographs of NHDF cells proliferated on the neat gelatin fibers mats and the 0.75-1.00% $AgNO_3$-loaded electrospun gelatin fibers mats at 7 days of cell culture; Mag = 500x and 3500x

Materials	500x	3500x
Polystyrene plate		
E-spun GT		
$AgNO_3$ 0.75 %-loaded e-spun GT fiber mats		
$AgNO_3$ 1.00%-loaded e-spun GT fiber mats		

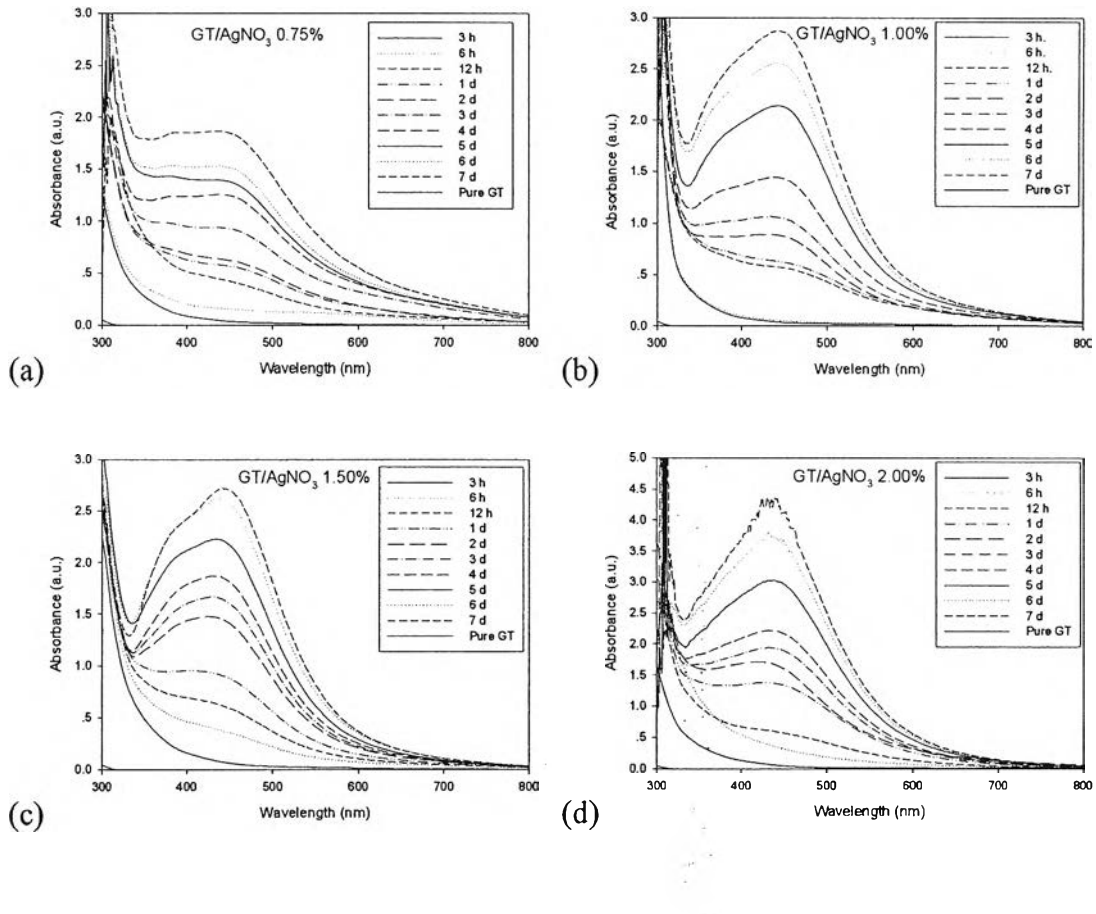
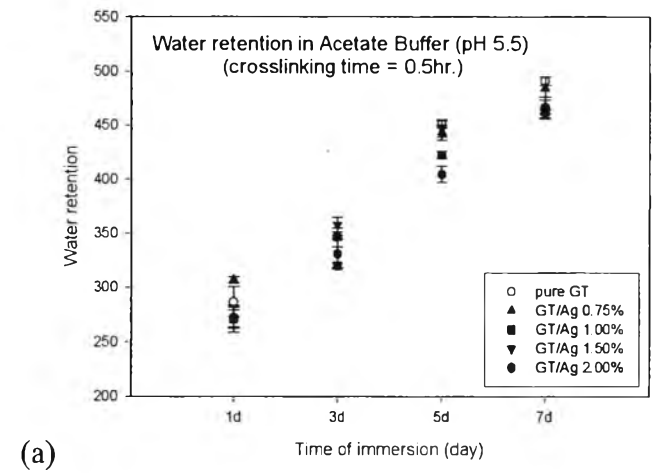
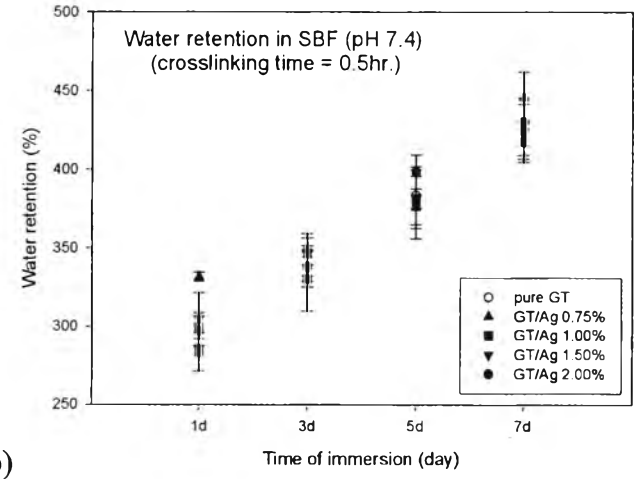


Figure 5.1 Variation in UV-visible absorption spectra of the base gelatin (GT) solution and the 0.75-2.00% $AgNO_3$-containing GT solutions that had been aged for different time intervals. The concentration of the base GT solution was 22 wt.% and the amount of $AgNO_3$ in the $AgNO_3$-containing GT solutions was 0.75-2.00 wt.% based on the weight of GT.

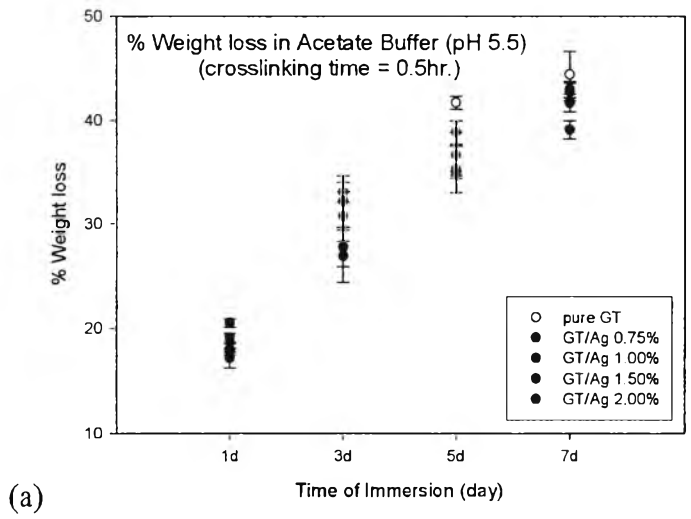


(a)

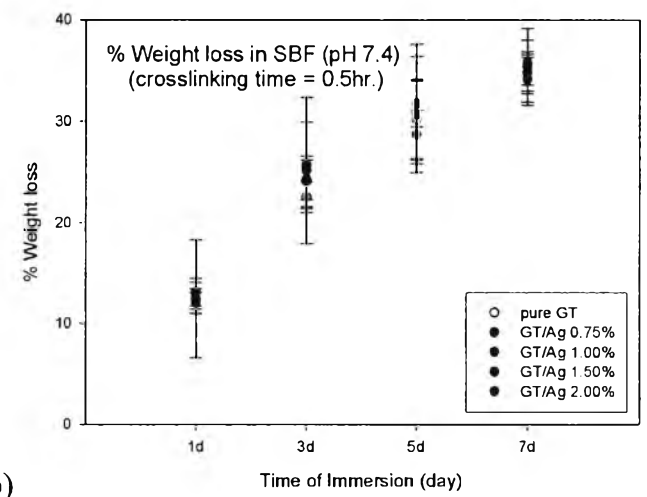


(b)

Figure 5.2 Water retention of the electrospun fiber mats from the base gelatin (GT) solution and the 12 h-aged 0.75-2.00% $AgNO_3$-containing GT solution, after having been cross-linked with moist vapor of glutaraldehyde for 0.5 h, as a function of submersion time in (a) acetate buffer solution (pH = 5.5) at 32°C, and (b) simulated body fluid (pH 7.4) at 37°C.

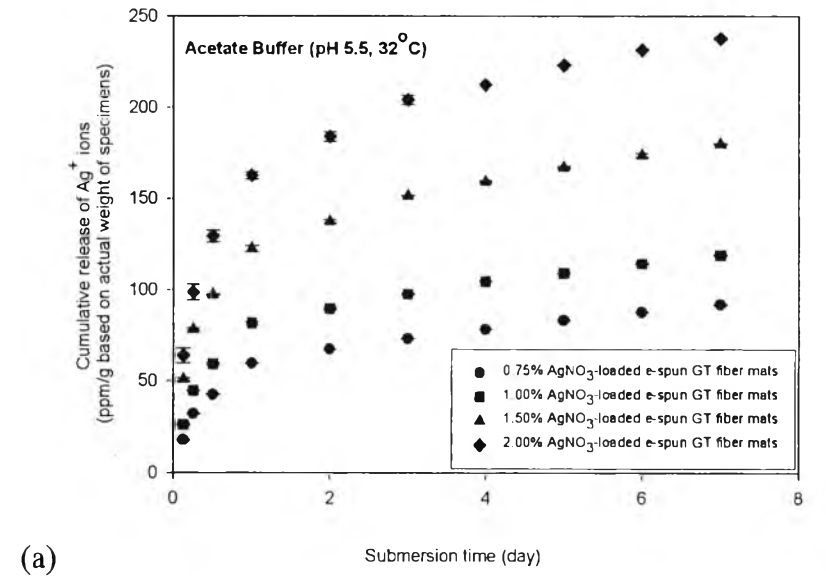


(a)

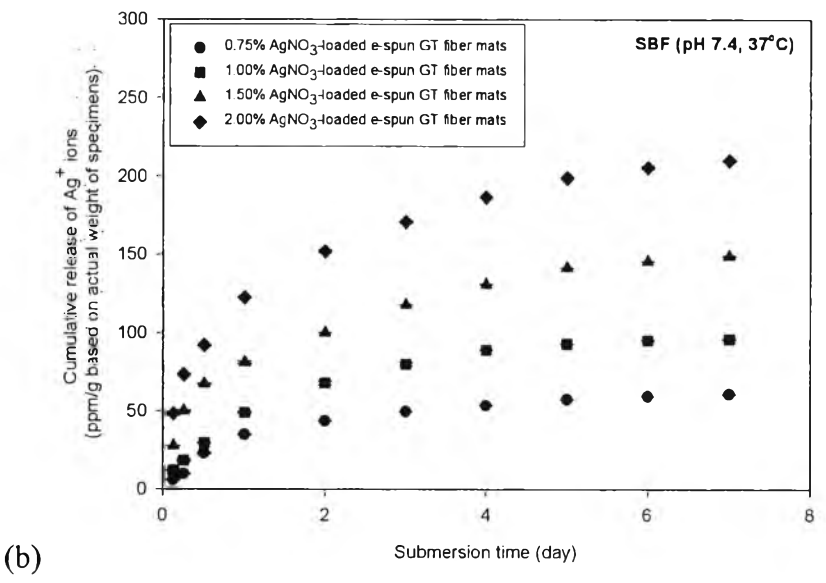


(b)

Figure 5.3 Weight loss of the electrospun fiber mats from the base gelatin (GT) solution and the 12 h-aged 0.75-2.00% $AgNO_3$-containing GT solution, after having been cross-linked with moist vapor of glutaraldehyde for 0.5 h, as a function of submersion time in (a) acetate buffer solution (pH = 5.5) at 32°C, and (b) simulated body fluid (pH 7.4) at 37°C.

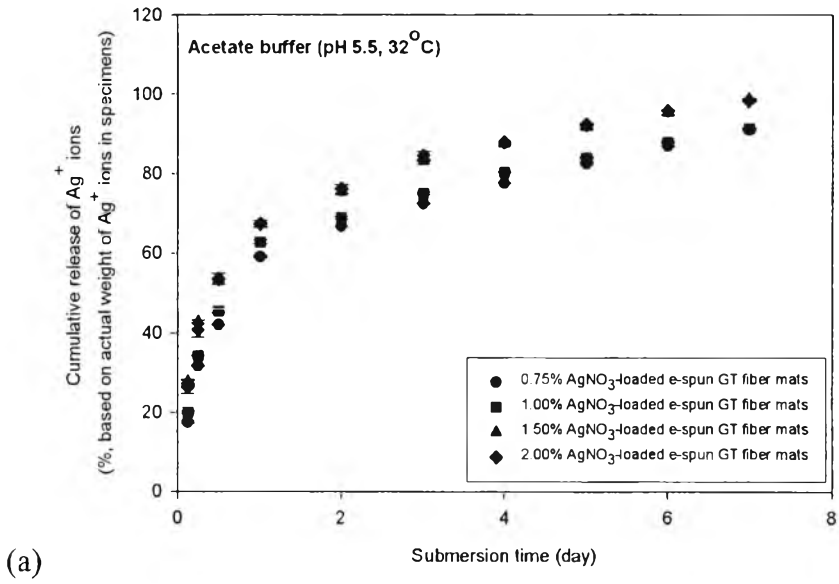


(a)

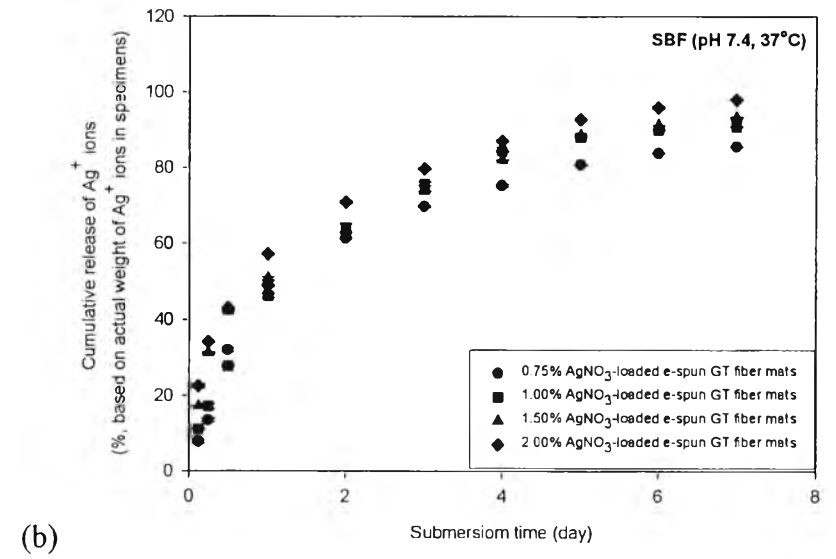


(b)

Figure 5.4 Cumulative release profiles of Ag^+ ions from 0.5 h-cross-linked nanoAg^0-containing e-spun gelatin (GT) fiber mat specimens reported as the concentration of Ag^+ ions released into the medium (in ppm of the medium) divided by the actual weight of specimens (in g) in two types of releasing medium, i.e., (a) acetate buffer (pH 5.5) at the skin temperature of 32°C, and (b) simulated body fluid (pH 7.4), at the physiological temperature of 37°C.

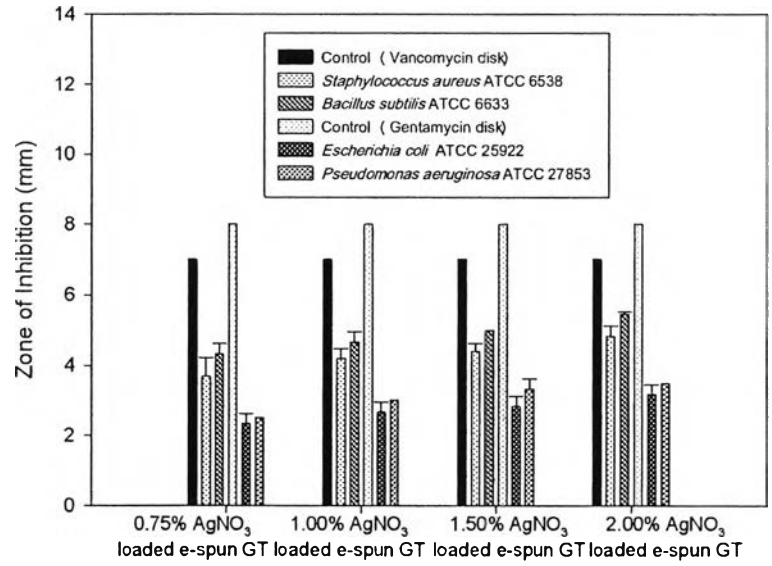


(a)

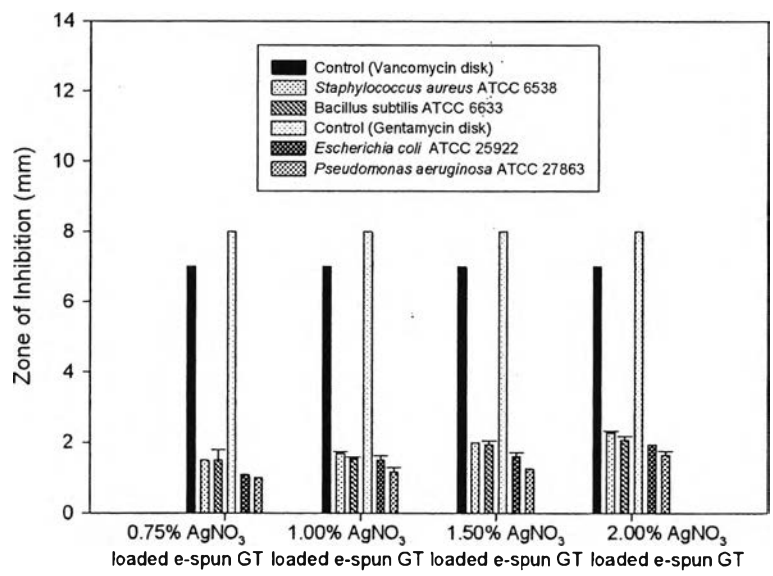


(b)

Figure 5.5 Cumulative release profiles of Ag^+ ions from 0.5 h-cross-linked nanoAg^0-containing e-spun gelatin (GT) fiber mat specimens reported as the percentage of the weight of Ag^+ ions released divided by the actual weight of Ag^+ in two types of releasing medium, i.e., (a) acetate buffer (pH 5.5) at the skin temperature of 32°C, and (b) simulated body fluid (pH 7.4) at the physiological temperature of 37°C.

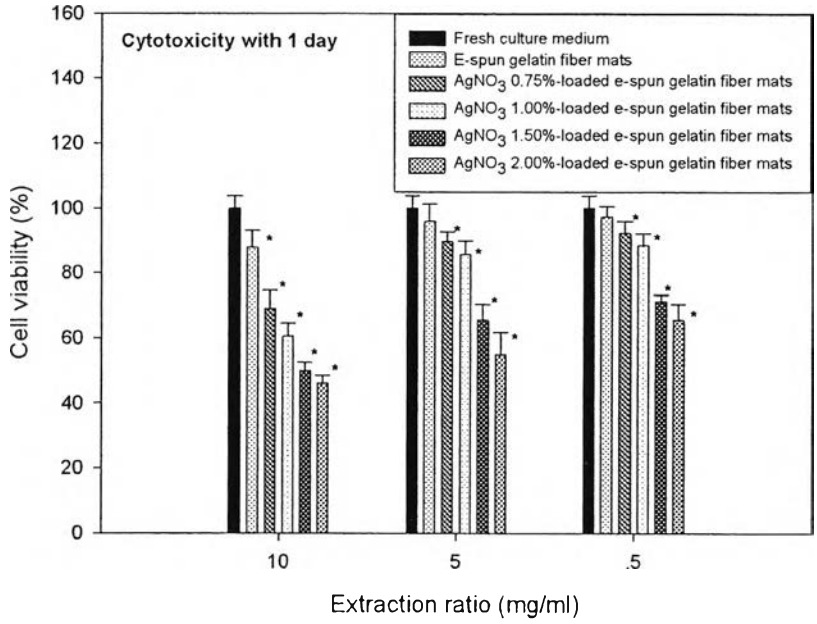


(a)



(b)

Figure 5.6 The influence of silver content on the antibacterial capacities of the 0.75-2.00% $AgNO_3$-loaded e-spun GT fibers mat against *Staphylococus aureus, Bacillus subtilis, Escherichia coli*, and *Pseudomonas aeruginosa* in (a) agar plates and (b) blood agar plates.



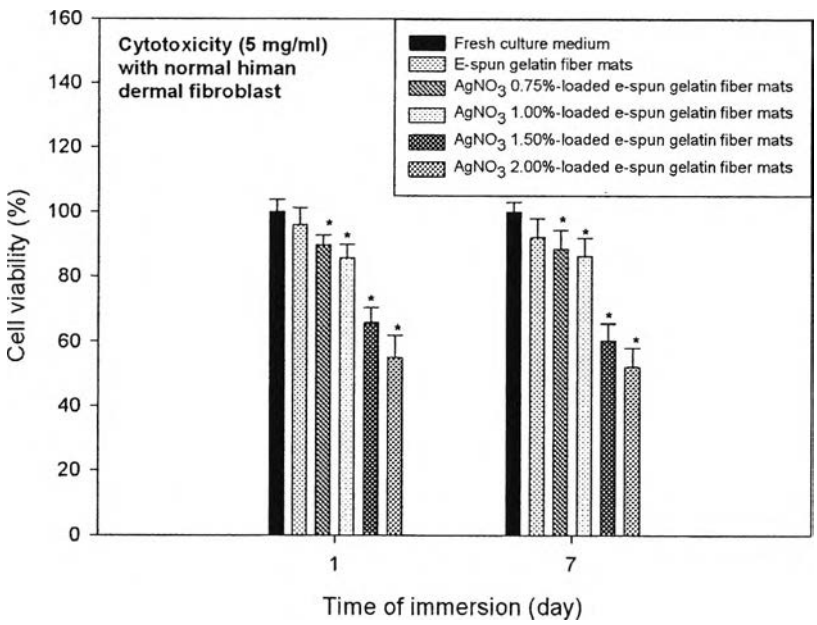


Figure 5.7 Indirect cytotoxicity evaluation of the neat gelatin and 0.75-2.00% $AgNO_3$-loaded electrospun fibers mats in comparison with viability of the cells that were cultured with fresh culture medium (n=3). (*) $p < 0.05$ compared with TCPS.