CHAPTER V

γ-RAY *IN SITU* SYNTHESIS OF SILVER NANOPARTICLES IN GELATIN HYDROGEL WITH ANTIBACTERIAL ACTIVITIES

5.1 Abstract

An antibacterial wound dressing material was successfully synthesized by ^{60}Co γ-ray irradiation of $AgNO_3$-loaded gelatin aqueous solution. Various amounts of $AgNO_3$ (i.e., at 0.25, 1, 3, 5 and 10%, based on the weight of gelatin) were loaded in 15% (w/v) gelatin solutions. These mixtures were subjected to γ-ray irradiation at various doses ranging from 20-80 kGy to form nAg-containing gelatin hydrogels. The formation of nAg was monitored by a UV-Vis spectrophotometer. The physical properties of these hydrogels including their gel fraction, swelling and weight loss behaviors were evaluated as a function of irradiation doses. With an increase in the irradiation dose to synthesize the hydrogels, the gel fraction was increased while the water retention and the weight loss were found to decrease. The potential to use nAg-containing gelatin hydrogels as wound dressings was assessed on investigation the release characteristic of the as-loaded silver, by evaluation of the indirect cytotoxicity using normal human dermal fibroblasts (NHDF) as well as its antibacterial activity against *Staphylococcus aureus* and *Methicillin-resistant Staphylococcus aureus*. The results showed that all nAg-containing gelatin hydrogels could inhibit the growth of the tested pathogens. With an increase in $AgNO_3$ content or contact time, the antibacterial activity of nAg-containing gelatin hydrogels was increased. These confirmed their applicability as antibacterial wound dressings of the nAg-containing gelatin hydrogels synthesized by γ-ray irradiation.

(**Key-words**: Hydrogel, Gamma radiation; antibacterial wound dressing; silver nanoparticles)

5.2 Introduction

Rapid and proper healing without infection is important in the treatment of burns (Bishara, S.A., *et al*, 2007). Using the wound dressing with antimicrobial activity is important in reducing the microbial around the wound surface. Once a wound becomes infected, healing is delayed (Percival, S.L., *et al*, 2005; Boateng, J.S., *et al*, 2007; Yates, C.C., *et al*, 2007). Generally, an effective wound dressing should maintain a moist environment by absorbing the exudates from the wounded area, provide adequate gaseous exchange, permit water evaporation at a certain rate, protect the wound from secondary infection, and also be elastic, biocompatible with tissues and blood, non-toxic and non-antigenic (Wu, P., *et al*, 1996; Kokabi, M., *et al*, 2007; Boateng, J.S., *et al*, 2007; Boateng, J.S., *et al*, 2008; Singh, J.S., *et al*, 2008). Based on these requirements, biocompatible polymeric hydrogel with antibacterial activity is one of the most promising materials for the burns (Babu, R., *et al*, 2006; Yudanoval, T.N., and Reshetov, I.V., 2006; Rattanaruengsrikul, V., *et al*, 2009).

The most effective antibacterial agents used in biomedical applications are silver-based materials. The application of silver-based compound as an antimicrobial agent has been well recognized (Cooper, R., 2004; Poon, V.K.M., and Burd, A., 2004; Singh, M., *et al*, 2008). This is generally attributed to the antimicrobial activities of the released silver ions (Cooper, R., 2004; Kumar, R., *et al*, 2005; Bhattacharya, R., and Mukherjee, P., 2008; Rujitanaroj, P., *et al,* 2008; Singh, M., *et al*, 2008). Presently, silver-based compound can be used to inhibit the growth of a wide variety of micro-organisms including yeast, fungi, viruses, and bacteria (Babu, R., *et al*, 2006; Kim, J.S., *et al*, 2007; AbdEl-Mohdy, H.L., and Ghanem, S., 2009); in diversity of applications especially in biomedical field (Babu, R., *et al,* 2006; Rujitanaroj, P., *et al,* 2008; Rattanaruengsrikul, V., *et al*, 2009).

The mechanism for the antibacterial action of silver-based materials can be one or more of the following processes. Silver ions may attach to the bacterial cell membrane, damage it and/or interfere various functions such as permeability and respiration. Additionally, silver ions may enter the cell in order to interact with structural proteins and preferentially bind with DNA bases leading to denaturation of

DNA and inhibition of replication. Furthermore, silver ions may inhibit adenosine triphosphate (ATP) synthesis by binding to an ATP synthase, finally leading to cell death (Bishara, S.A., *et al*, 2007; Shrivastava, S. *et al*, 2007; Rujitanaroj, P., *et al,* 2008; Rattanaruengsrikul, V., *et al*, 2009). However, the antibacterial property of silver-based compound depends on their dimensions, where the smaller particles with a larger surface to volume ratio provided a greater antibacterial effect (Mahapatra, S.S., *et al*, 2008). Therefore, much attention has been paid to the development of the method to synthesize the stable silver nanoparticles (nAg) with controllable shape, size, and size distribution in the suitable matrices (Rujitanaroj, P., *et al,* 2008; Rattanaruengsrikul, V., *et al*, 2009).

Numerous approaches have been used to synthesize the nAg. Some of these are chemical reduction, (Kim, J.S., *et al*, 2007; Rujitanaroj, P., *et al,* 2008; Rattanaruengsrikul, V., *et al*, 2009) photochemical (Li, H.X., *et al*, 2000; Scire, S., et al, 2009), microemulsion (Zhang, W., *et al*, 2006; Zhang, W., *et al*, 2007), magnetron sputtering (Chandra, R., *et al*, 1999; Chen, Q., *et al*, 2008), electrochemical synthesis (Starowicz, M., *et al*, 2006; Zhou, M., et al, 2006), sonochemical method (Soloviev, M. and Gedanken, A., 2011), laser ablation (Verma, R.K., *et al*, 2010), supercritical liquid (Kamrupi, I.R., *et al*, 2010), etc. However, most of the above methods require the use of high temperature and/or high pressure many organic solvents and toxic reducing agents like DMSO (Mahapatra, S.S., *et al*, 2008), formamide (Sarkar, A., *et al*, 2010), N,N-dimethyl formamide (Rujitanaroj, P., *et al*, 2010), hydrazine (Kim, J.S., *et al*, 2007) and sodium borohydride (Maneerung, T., *et al*, 2008), etc. All these chemicals are highly reactive and pose potential environmental and biological risks (Huang, H., and Yang, X. 2004; Hebeish, A.A., *et al*, 2010).

During the last few decades, the use of the gamma-radiation-induction technique to synthesize the nAg has gained extensive attention. This method involves the reduction of silver ions in an aqueous solution by hydrated electrons and secondary radicals which are the products of water radiolysis (Krklješ, A., *et al*, 2007). Not only the radiation technique has induced the formation of silver ions reduction but also the cross-linking of polymer network to form hydrogel structure (Rosiak, J.M., *et al*, 1999; Bhattacharya, A., 2000; Chmielewski, A.G., *et al,* 2007; Cataldo, F. *et al*, 2008). In fact, there are a number of methods used for formation of

hydrogels, however the radiation synthesis seems to be the most useful for medical purposes (Rosiak, J.M., *et al*, 1999; Bhattacharya, A., 2000). Since the as-irradiated hydrogels are sterilized, highly purified, free of monomers, initiators and any other additives (Rosiak, J.M., *et al*, 1995; Bhattacharya, A., 2000; Chmielewski, A.G., *et al*, 2007; AbdEl-Mohdy, H.L., and Ghanem, S., 2009). The versatile advantages of this method are (1) the process is simple, clean and easy to control (2) controllable reduction of silver ions can be manipulated without using reducing agents (3) no wastes, byproducts and impurities are introduced because the process does not need to use any cross-linkers or initiators (4) the method provides sterilized product with high degree of assurance level (5) the technology is environment friendly since it leaves no residue or pollutant in the environment and (6) the metal/polymer nanocomposites hydrogels were prepared simultaneous in aqueous solution through single step (Rosiak, J.M., *et al*, 1999; Bhattacharya, A., 2000).

In the present contribution, we describe a versatile and effective technique for the preparation of the metal/polymer nanocomposites hydrogels. Gelatin as a matrix component is selected because it is a natural hydrogel-forming biopolymer derived from hydrolysis of native collagen (Olsen, D., *et al*, 2003; Young, S., *et al* 2005). Due to the unique functionality of gelatin, it has been used in a wide variety of applications including in pharmaceutical and medical fields (Olsen, D., *et al*, 2003; Young, S., *et al* 2005; F. Cataldo, *et al*, 2008; Vlierberghe, S.V., *et al* 2008) as ingredients in drug formulations (Nazzal, S., and Wang, Y., 2001), carriers for delivery of drugs (Konishi, M., *et al*, 2005) or other therapeutic substances (Fukunaka, Y., *et al*, 2002; Hori, K., *et al*, 2002) and, especially, as wound-dressing materials (Rujitanaroj, P., *et al*, 2008; Rattanaruengsrikul, V., *et al*, 2009).

In the present contribution, the nAg-containing gelatin hydrogels were fabricated by gamma irradiation of $AgNO_3$-loaded gelatin solutions. The aim of the study is to investigate the potential application of the nAg-containing gelatin hydrogels as antibacterial wound dressings. The physical properties such as gel fraction, swelling and weight loss behavior of the obtained gelatin hydrogels were evaluated as a function of irradiation doses. The release characteristic of as-load silver, the indirect cytotoxicity as well as the antibacterial evaluation of silver nAg-containing gelatin hydrogels were investigated.

5.3 Experimental Details

5.3.1 Materials

Gelatin powder, type A (obtained from porcine skin; 170-190 Bloom) was purchased from Fluka (Switzerland). Analytical grade silver nitrate ($AgNO_3$; 99.998% purity) was purchased from Fisher Scientific (USA). All other chemicals used were of analytical reagent grade and were used as received without further purification.

5.3.2 Preparation of Neat Gelatin and nAg-containing Gelatin Hydrogels

Gelatin powder was dissolved in distilled water to obtain 15 wt% solution, and was stirred at a temperature of 40 °C for 1 h. A metered weight of $AgNO_3$ (0-10% wt based on the weight of the gelatin powder) was then added into the as-prepared gelatin solution. Further stirring was used to homogenize the solution. The as-prepared gelatin containing silver ion solution was poured gently into hot-sealed 4 × 7.5 inch nylon bag (35 g). The samples were irradiated with γ-rays from a ^{60}Co Gammacell irradiator at Thailand Institute of Nuclear at dose rates of 1 kGy/h at room temperature. Gamma irradiation was performed at the fixed radiation dose of 20, 40, 60 and 80 kGy.

5.3.3 Measurement of Gel Fraction, Swelling and Weight Loss Behavior of the Neat and nAg-containing Gelatin Hydrogels

The neat and nAg-containing gelatin hydrogels obtained by irradiation were cut into circular disc with diameter of 1.5 cm and dried at 50 °C until reached constant weight. The gel fraction of these hydrogels was estimated by measuring their insoluble part after extraction in distilled water at the temperature of 50 °C for 2 h with violent shaking of 100 rpm. The insoluble specimens were then dried at 50 °C until reached constant weight. The gel fraction of these specimens was calculated according to the following equation:

$$Gel\ fraction\ (\%) = \frac{M_e}{M_i} \times 100 \qquad (1)$$

where M_e is the weight of dried specimens after extraction and M_i is the initial weight of the specimens.

To study the swelling and weight loss behavior, the pre-dried neat gelatin hydrogels (circular disc; diameter of 1.5 cm) were submerged in distilled water (pH 6.9), phosphate buffer solution (PBS; pH 7.4) and simulated body fluid (SBF; pH 7.4) at the physiological temperature of 37 °C. Procedures for the preparation of PBS and SBF are given in Supplementary data. The percentage of swelling and weight loss of these specimens were calculated according to the following equations:

$$Swelling\ (\%) = \frac{(M - M_i)}{M_i} \times 100 \quad (2)$$

$$Weight\ loss\ (\%) = \frac{(M_i - M_d)}{M_i} \times 100 \quad (3)$$

where M is the weight of each swollen specimen after the excess surface water was removed with filter paper 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 Measurement of Moisture Retention Capability

The moisture retention capability of the gelatin hydrogels was measured by using the same method of Min Wang and coworkers (Wang, M., *et al* 2007). The circular disc; diameter of 4 cm with thickness of ~2.5 mm of the neat and nAg-containing gelatin hydrogels were placed on PTFE plates, and then heated in an oven with the controlled temperature of 37 °C. Moisture retention capability of these hydrogel specimens was measured by the water losing rate and the ratio of water holding in the hydrogel specimens (R_h), which was calculated as following equation:

$$R_h(\%) = \frac{M_t}{M_0} \times 100 \quad (4)$$

where M_0 is the initial weight of the hydrogel specimens and M_t is the weight of each specimen at different intervals time point.

5.3.5 Measurement of Water Vapor Transmission Rate

Water vapor permeability of the hydrogel specimens was measured according to the ASTM E96-95 (1995) with some modifications. Distilled water (7.5 mL) was added into the glass cup (internal diameter of 2 cm) and the circular disc; diameter of 2.3 cm with thickness of ~2.5 mm of the neat and nAg-containing gelatin

hydrogels were covered on the open area of the cups. The cups were incubated at 25 °C and 50% relative humidity. After weighing the initial weight, the weight was measured every 2 h until 12 h. Weight loss of the cups with time was measured and a linear regression analysis was performed to calculate the slope. Water vapor transmission rate (WVTR; $g/m^2/d$) of the hydrogel specimens was calculated by dividing the slope by the open area of the cup.

5.3.6 Characterization of nAg Formation

The existence of the as-formed nAg in the nAg-containing gelatin hydrogels was confirmed by monitoring the surface plasmon absorption band using a Shimadzu UV-2550 UV–visible spectrophotometer (USA). Ethanolic extracted of the nAg-containing gelatin hydrogel was used to examine the nAg in the nAg-containing gelatin hydrogels by a JEOL JEM-2100 transmission electron microscope (TEM; Japan), and an Oxford 2000 energy dispersive X-ray (EDX; Japan) facility of a Phillips PW 2400 X-ray fluorescence spectroscope.

5.3.7 Loading Capacity and Release Characteristic of As-loaded Silver From the nAg-containing Gelatin Hydrogels

Prior to the release assay, the actual amount of as-loaded silver in the nAg-containing gelatin hydrogel specimens (circular disc; 2.8 cm in diameter) need to be determined. The specimens were dissolved in 50 mL of 50% nitric acid (HNO_3) solution. After that, each of the solutions was quantified for the amount of silver by a Varian SpectrAA-300 atomic absorption spectroscope (AAS; Japan). The results were reported as average values (n = 3). The release characteristics of as-load silver from the nAg-containing gelatin hydrogel specimens (circular disc; 2.8 cm in diameter) was assessed in 25 mL of PBS as the releasing medium at the physiological temperature of 37 °C. At a specified immersion period ranging between 20 min to 10 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 as-loaded silver in the specimens.

5.3.8 Antibacterial Evaluation

The antibacterial activity of the neat and nAg-containing gelatin hydrogels was tested against aerobic bacteria commonly found on burn wounds: e.g., *Staphylococcus aureus* (Gram-positive; ATCC 25023), and *Methicillin-resistant Staphylococcus aureus* (MRSA: Gram-positive; ATCC 20645).

5.3.8.1 Disc Diffusion Test Method.

The assessment was conducted based on the disc diffusion method of the US Clinical and Laboratory Standards Institute (CLSI). The circular discs; diameter of 8 mm with thickness of 2.5-3 mm of the neat and nAg-containing gelatin hydrogels were used against vancomycin as the control antibacterial drug. Each of the specimens and the control drugs were placed on Difco™ Mueller Hinton agar in a Petri dish, and then incubated at 37 °C for 24 h. If inhibitory concentrations were reached, there would be no growth of the microbes, which could be seen as clear zone around the disc specimens.

5.3.8.2 Dynamic Shakes Flask Test Method.

Quantitative procedure for evaluation of the degree of antibacterial activity of the neat and nAg-containing gelatin hydrogels was provided according to the ASTM E 2149-01 (standard test method for determining the antimicrobial activity of immobilized antimicrobial agents under dynamic contact conditions). This test method is designed to evaluate the resistance of non-leaching antimicrobial treated specimens to the growth of microbes under dynamic contact conditions.

2 g of gelatin hydrogels with and without nAg were cut into small pieces and sterilized by autoclave at 121 °C for 15 min before used. The specimens were subjected to constant agitation in a concentrated bacterial suspension (10^5 CFU/mL, 50 mL) at 37 °C. At 0, 20, 40, 60 min 2, and 3 h contact times, 1 mL of the suspension was withdrawn and made serial dilutions with sterilized normal saline and spiral on plates (in triplicate) on nutrient agar. These palates were brought into incubator at 37 °C for 24 h. Finally, the colonies on agar plate were photographed and count to evaluate the antibacterial activity. The number of viable bacteria present in the suspension was determined and the percentage reduction at specific period of contact time was calculated base on the following formula:

$$\text{Reduction}\,(\%) = \frac{(B - A)}{B} \times 100 \qquad (5)$$

where A is the number of bacteria recovered from the incubated treated test specimen (the nAg-containing gelatin hydrogels) after 37 °C for 24 h and B is the number of bacteria recovered from the incubated untreated control specimen (the neat gelatin hydrogels without nAg) after incubation at 37 °C for 24 h.

5.3.9 Indirect Cytotoxicity Evaluation

The indirect cytotoxicity evaluation of both the neat and nAg-containing gelatin hydrogels was conducted in adaptation from the ISO 10993-5 standard test method in a 96-well tissue-culture polystyrene plate (TCPS; NunclonTM, Denmark) using normal human dermal fibroblasts (NHDF; seventh passage) as reference. 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 sterilized specimens (autoclaved small pieces that were cut from the hydrogel samples and then dried in the oven) were immersed in serum-free medium (SFM; containing DMEM, 1% L glutamine, 1% lactabumin, and 1% antibiotic and antimycotic formulation) for 24 h in incubator to produce extraction media concentration of 10 and 20 mg/mL. NHDF were separately cultured in wells of TCPS at 8,000 cells/well in serum-containing DMEM for 24 h to allow cell attachment. The cells were then starved with SFM for 24 h. After that, the medium was replaced with an extraction medium and cells were re-incubated for 24 h. 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 for a 96-well

TCPS. Secondly, the plate was incubated for 2 h at 37 °C. The solution was then aspirated and 100 μL/well of DMSO was added to dissolve the formazan crystals. Finally, after 3 min of rotary agitation, the absorbance at the wavelength of 570 nm representing the viability of the cells was measured using a SpectraMax M2 microplate reader (Molecular Devices, USA).

5.3.10 Statistical Analysis

Data were presented as means ± standard errors of means. Statistical analysis was carried out by the one-way analysis of variance (one-way ANOVA) and Scheffe's post hoc test in SPSS 11.5 for Windows software (SPSS). The statistical significance was accepted at $p < 0.05$.

5.4 Results and Discussion

5.4.1 Preparation of Neat and nAg-containing Gelatin Hydrogels

When aqueous solution of gelatin containing Ag^+ ions is subjected to gamma irradiation, the reactive species from the radiolysis of water are produced. These species are hydrated electron; e^-_{aq}, hydroxyl radicals; $OH^•$, and a small amount of hydrogen atom (Rosiak, J.M., et al, 1999; Krklješ, A., *et al*, 2007; Varshney, L. 2007) as shown in equation 6. Then they may attract Ag^+ ions and gelatin molecules in order to reduce Ag^+ ions to neutral Ag^0 atoms (Chen, P., *et al,* 2007) and to cross-link gelatin (Hara, M. 2006), respectively to generate gelatin–nAg nanocomposites.

The radiation-induced reduction of Ag^+ ions could be taken place during the irradiation of the aqueous solution of gelatin containing Ag^+ ions. It has been reported Ag^+ ions are reduced by hydrated electrons; e^-_{aq}; produced from water radiolysis, to form the neutral Ag^0 atoms as a primary reduction products (Zhu, Y., et al, 1997; Temgire, M.K., and Joshi, S.S., 2004; Krklješ, A., *et al*, 2007; Chen, P., *et al,* 2007) (equation 7). Then these neutral Ag^0 atoms undergo further aggregation to progressively larger clusters via various growth processes leading to the formation of nAg at last (Xu, X., et al, 1998; Shin, H.S., *et al*, 2004; Chen, P., *et al,* 2007) (equation 8-10). The as-formed nanoparticles via gamma irradiation are capped within the gelatin network. The mechanism of nAg formation by irradiation-reduction process can be written as follow.

$$H_2O \longrightarrow OH^{\bullet}, e^{-}_{aq}, H^{\bullet}, H_2O_2, H_2, H^{+}, \ldots \quad (6)$$

$$Ag^{+} + e^{-}_{aq} \longrightarrow Ag^{0} \quad (7)$$

$$Ag^{0} + Ag^{+} \longrightarrow Ag_2^{+} \quad (8)$$

$$Ag_2^{+} + Ag^{+} \longrightarrow Ag_3^{+} \quad (9)$$

$$\dashrightarrow \dashrightarrow Ag_n \text{ (aggregation to form Ag nanoparticles)} \quad (10)$$

The radiation-induced cross-linking of gelatin chains as the following reaction (Ila, D., *et al*, 2000) is known to be generally initiated by hydroxyl radicals (Kojima, T., *et al,* 2004). The hydroxyl radicals can abstract hydrogen atoms from the gelatin chains and thus produce a carbon-centered radical in gelatin chain (see equation 11). Finally cross-linked gelatin hydrogels are formed by the reaction of chain propagation of macroradicals (see equation 12 and 13).

$$OH^{\bullet} + GT \longrightarrow GT^{\bullet} + H_2O \quad (11)$$

$$GT^{\bullet} + GT \longrightarrow GT\text{-}GT^{\bullet} \quad (12)$$

$$GT\text{-}GT^{\bullet} + {}^{\bullet}GT\text{-}GT \longrightarrow \text{Cross-linked GT hydrogels} \quad (13)$$

The obtained neat and nAg-containing gelatin hydrogels show good appearance with the hydrogels characteristics of moisture, softness, elasticity and pleasant to touch. It can be seen that the neat gelatin hydrogel shows good transparency while nAg-containing gelatin hydrogels exhibit slightly brown to dark brown with increasing the as-loaded silver due to the presence of nAg (Thomas, V., *et al*, 2007; Rujitanaroj, P., *et al,* 2008; Rattanaruengsrikul, V., *et al*, 2009). Gamma radiation has been recognized as one of suitable tools for the formation of nAg/hydrogel nanocomposites within single step. These hydrogels those were prepared by irradiation technique possess sterilization. The irradiation technique is clean and environmental friendly process since no necessity to use any cross-linkers or reducing agents possibly harmful and difficult to remove and no waste or byproducts are formed. This makes irradiation technique is the promising method useful for medical purposes.

5.4.2 Measurement of Gel Fraction, Swelling and Weight Loss Behavior of Neat and nAg-containing Gelatin Hydrogels

5.4.2.1 Gel Fraction

Irradiation of $AgNO_3$-loaded gelatin aqueous solution results in simultaneous cross-linking of gelatin to form a three-dimensional polymer network, meanwhile silver ions undergo reduction into Ag^0 eventually lead to the formation of nAg trapped inside the gelatin hydrogels.

0-10% $AgNO_3$-loaded gelatin aqueous solutions that had been exposed to gamma irradiation doses of 20, 40, 60 and 80 kGy were extracted with water of 50 °C for 2 h under vigorous shaking to evaluate the effect of irradiation dose on the gel fraction of these as-prepared hydrogels. The gel fraction of the as-prepared gelatin hydrogels as a function of irradiation dose were shown in Figure 5.1.

For any types of hydrogels, it was observed that gel fraction increased with increasing irradiation dose. The gel fraction increases rapidly at low dose and slightly increases above 60 kGy. The high gel fraction of hydrogels at high irradiation dose was due to higher degree of cross-linking of polymer network.

At a given radiation dose, the gel fraction of the hydrogels decreased with an increase in the percentage of as-loaded silver ions that had been loaded into the gelatin solutions. That would result from the mechanism of nAg formation via irradiation synthesis. Upon γ-irradiation, the water undergoes radiolysis to generate hydrated electrons, which reduced the Ag+ ions to form the nAg. The cross-linking of gelatin is the following reaction (Ila, D., *et al*, 2000). It was result from the hydroxyl radicals from the radiolysis of water react efficiently with gelatin molecules to form gelatin network. At a given radiation dose, partial energy that had given to the $AgNO_3$-loaded gelatin aqueous solution was used to react with Ag^- to form Ag^0, then cross-linking process of gelatin will start later with excess energy. The more Ag^+ ions were loaded, the more energy were used to reduce Ag^+ ions to Ag^0 consequently the less energy remained were used to cross-link polymer network. Therefore increasing as-load silver in the gelatin solution resulted in the lowering of the gel fraction of hydrogels.

5.4.2.2 Swelling and Weight Loss Behavior

The measurements for the water swelling and the weight loss were used to assess the extent of the cross-linking of neat gelatin hydrogels that had been irradiated by the gamma ray with various doses. Three types of aqueous medium were used for these purposes: distilled water (pH 6.9), phosphate buffer solution; PBS (pH 7.4), and simulated body fluid; SBF (pH 7.4). The results are graphically shown in Figure 5.2.

At a given radiation dose, the water swelling of the neat gelatin hydrogels, in any type of the medium, increased with the submersion time then became saturated within 24 h and finally they have undergone a series of disintegration after they had been submerged in the medium for about 1-10 d. At the same time, it was observed that the %swelling, at any given time point, decreased with an increase in the radiation dose used to synthesize the hydrogels. This was due to the higher irradiation dose applied to the gelatin solutions, leading to greater cross-link density of the as-prepared gelatin hydrogels attained (Savas, H. and Guven, O., 2002).

For the hydrogels that had been submerged in the distilled water for 24 h, the amounts of the swelling were found to decrease from about 2860% at 20 kGy irradiated to about 1230% at 80 kGy irradiated. In the PBS, such values decreased from about 1452% at 20 kGy irradiated to about 822% at 80 kGy irradiated. In the SBF, the values decreased from about 1490% at 20 kGy irradiated to about 779% at 80 kGy irradiated. Evidently, the amount of the water retained in the hydrogels after they had been submerged in distilled water was the highest.

On the other hand, the loss in the weight of the hydrogels, at any given radiation dose and in any type of the medium, increased with the submersion time within 10 h and then became increase further afterward. Simultaneously, it was found that, at any given time point, the values decreased with an increase in the radiation dose. Specifically, for the hydrogels that had been submerged in the distilled water for 24 h, the percentages of the weight loss decreased from about 33% at 20 kGy irradiated to about 17% at 80 kGy irradiated. In PBS, such values decreased from about 31% at 20 kGy irradiated to about 13% at 80 kGy irradiated. In the SBF, the values decreased from about 24% at 20 kGy

irradiated to about 10% at 80 kGy irradiated. Evidently, the greatest extent of the weight loss of the hydrogels occurred when they were submerged in the distilled water.

5.4.3 Number-average Molecular Weight of Chain Segments Between Cross-linking Points and Cross-link Density

The water swelling data of the hydrogels after having been submerged in distilled water at ambient temperature for 1 d were used to calculate the number-average molecular weight of chain segments between cross-linking points (Mc) and the cross-link density (Ve) of the as-prepared nAg-containing gelatin hydrogels. The results of such calculations are illustrated in Figure 5.3. Apparently, an increase in the irradiation dose caused the Mc values of the neat gelatin hydrogel to decrease from about 16320 g/mol at 20 kGy of irradiation dose to about 5152 g/mol at 80 kGy of irradiation dose. These corresponded to a monotonous increase in its Ve values from about 4.98×10^{19} #/cm^3 at 20 kGy of irradiation dose to about 15.88×10^{19} #/cm^3 at 80 kGy of irradiation dose. Recently, we also showed that the Ve values of the neat gelatin hydrogels those had been chemically cross-linked with glutaraldehyde aqueous solution (mixed at various concentrations of 0.5, 1, 3, 5, 7, 9 μl per ml of gelatin solution) were increase, while the Mc values were decrease by increasing in the concentration of glutaraldehyde (Rattanaruengsrikul, V., *et al*, 2009). Apparently, the obtained results agreed well with these previous reports, however the chemically cross-link provides greater degree of cross-linking than physically method.

The increasing in the Mc and the reducing in the Ve values of the nAg-containing gelatin hydrogels at any irradiation dose were observed when $AgNO_3$ were loaded in the gelatin hydrogels. Such the values were dependence on the concentration of as-loaded $AgNO_3$. This indicated that the cross-link density of the nAg-containing gelatin hydrogels decreased with an increase in the amount of as-loaded $AgNO_3$. Because of the value of energy absorption of Ag was higher than that of gelatin, therefore the reduction of Ag^+ ions by hydrated electron had occurred first then the cross-linking of gelatin network by hydroxyl radical has been taken place later. Higher energy was consumed at higher concentration of $AgNO_3$ loaded and the less remained energy was used to generate the gelatin hydrogels. This result of lower

cross-link density at the higher level of as-loaded silver at any irradiation doses corresponds to the gel fraction and the swelling and weight loss behavior of the nAg-containing gelatin hydrogels at any irradiation dose.

5.4.4 Measurement of Moisture Retention Capability of Neat and nAg-containing Gelatin Hydrogels

The moisture retention capacity is one of the important factors of wound dressing to maintain a moist environment over the wound area. Moisture retention capacity of nAg-containing gelatin hydrogels that had been irradiated at 60 kGy was investigated as a function of time and is calculated as the water losing rate and the ratio of water holding in the hydrogels.

As shown in Figure 5.4 the water inside all hydrogels that had been irradiated at 60 kGy tended to gradually evaporate continuously over the time with the water losing rate (slope of the graph) in the similar range of 7.1×10^{-2} to 7.7×10^{-2} g/h as shown in Table 5.1. The water losing rate of nAg-loaded gelatin hydrogels that had been irradiated at 60 kGy is slightly greater than the neat gelatin hydrogel consequently the ratio of water holding in hydrogel at any given time point is slightly smaller than the neat gelatin hydrogel. All hydrogel specimens take about 50 h to lose most of their water. Figure 5.4 shows the ratio of water holding in the neat and the nAg-containing gelatin hydrogel specimens at various time intervals. At 4 h. the ratio of water holding in the neat gelatin hydrogel specimens ($R_{h,\ 4h}$) was 70.08 % and reduced to 58.94% and 37.96% at 6 h and 10 h, respectively. The value of $R_{h,\ 4h}$ of 1, 3, 5 and 10% nAg-containing gelatin hydrogels were 68.74, 68.75, 66.79 and 59.04% respectively and reduced to 52.16, 53.17, 53.86 and 47.65% at 6 h. respectively then those value of $R_{h,\ 10h}$ of 1, 3, 5 and 10% nAg-containing gelatin hydrogels were reduced further to 30.24, 32.88, 32.76 and 27.83%, respectively. Wang, M. *et al.* (2007) reported that the PVP/CMC blend hydrogels have the similar moisture retention capability regardless with their cross-linking density and composition since the moisture retention capability of all hydrogels was linear with the water losing rate of 5.0×10^{-4} g/min, and the $R_{h,\ 4h}$ value was about 69%. In his account (Wang, M. *et al.*, 2007), the comparison between PVP/CMC hydrogel and a commercial hydrogel wound dressing was performed using the PVP/CMC hydrogel at a fixed ratio of 6:4 irradiated with gamma ray of 25 kGy in comparison with a

commercial hydrogel dressing mainly containing PVA obtained from Changchun JA Biotech. Co. Ltd., China. The results showed that the two kinds of dressings had similar rate of losing water, which was about 5.0×10^{-4} g/min. And the $R_{h,\ 6h}$ of the PVP/CMC hydrogel was 54.5% which was comparable to the commercial hydrogel dressing of 51.7%. Apparently, the neat and the nAg-containing gelatin hydrogels that had been irradiated at 60 kGy showed the close value to the previous reports.

It is beneficial to use hydrogel dressing to maintain moist for wound environment. A balanced moist surface facilitates the action of growth factors and cytokines, thus promoting cellular growth and collagen proliferation leading to the establishment of a provisional wound matrix (Boateng, J.S., *et al*, 2007; Boateng, J.S., *et al*, 2008; Okan, D., et al, 2007; Ovington, L.G., 2007).

5.4.5 Measurement of Water Vapor Transmission Rate (WVTR) of Neat and nAg-containing Gelatin Hydrogels

Neat and nAg-containing gelatin hydrogels; thickness of ~2.5 mm were measured the water vapor transmission rate at controlled temperature of 25 °C and 50 % R_h. Because they were irradiated at the same dose of 60 kGy so the WVTR of all samples was in the same range of 2835-3039 g/m^2/d as shown in Table 5.1. It was well known that the WVTR depended on thickness of specimens however; the hydrogels should tailor-made at the suitable thickness for appropriate use with any wound types. Lamke, L.O. *et al.* (1977) reported the value of the water evaporate from a normal skin, a first degree burn and a third degree burn is about 204±12, 279±26 and 5138±202 g/m^2/day, respectively. The effectual wound dressing should maintain a moist environment upon absorption of the wound exudates by prevention of the excessive dehydration as well as avoiding of the exudates accumulation (Balakrishnan, B., *et al.* 2005). It has been recommended that a rate of 2000–2500 g/m^2/day would provide adequate level of moisture without risking wound dehydration (Queen, D., *et al.* 1987). Wound dressings available in market such as Geliperms (Geistlich Ltd., Switzerland) and Vigilons (Bard Ltd., Crawley, UK) were found to have a WVTR of 9009±319 and 9360±34 g/m^2/day, respectively, and thus acts as water-free surface (Wu, P., *et al.* 1995). Such high WVTR would lead to total dehydration of the wound surface enabling the dressing adhere to the wound. The as-prepared gelatin hydrogels showed a value close to the range appropriate to maintain

a proper fluid balance on the wound bed. This slightly higher value results from microporous morphology inside the hydrogels. Therefore the gelatin hydrogels could be applied with the high exudates wound because the exudates could be absorbed and evaporated through the hydrogels easily in order to prevent the accumulation of wound exudates.

5.4.6 Characterization of nAg in the nAg-containing Gelatin Hydrogels

The formation of Ag nanoparticles within the gamma irradiated gelatin hydrogels could be assessed from the presence of the characteristic surface Plasmon band at around 410–430 nm (Liu, F.K., *et al*, 2007; Liu, Y., *et al,* 2009; Rujitanaroj, P., *et al,* 2008; Rattanaruengsrikul, V., *et al*, 2009) in the UV-vis absorption spectrum. Figure 5.5 shows the UV–vis absorption spectra of the neat and nAg-containing gelatin hydrogels irradiated at 60 kGy. There is no UV–vis absorption of any kind observed for the neat gelatin hydrogel base. Until loading $AgNO_3$ into gelatin solution and irradiated with the gamma ray then the hydrogels were formed together with nAg synthesized, so the characteristic peak of nAg was observed. The higher intensity of the characteristic peak of Ag nanoparticles was obtained when increase the percentage of as-loaded silver in the hydrogels from 1 to 10%. This suggests the formation of nAg with higher yields (Liu, F.K., *et al*, 2007). Moreover, increasing of Ag-loaded, from 1 to 10%, the corresponding maximum wavelength of the absorption spectrum was shift from 460 to 420 nm. This implies the formation of Ag nanoparticles with smaller sizes (Liu, F.K., *et al*, 2007).

TEM studies (Figure 5.6) confirmed that Ag nanoparticles are not agglomerated, well-dispersed and almost spherical in shape with particles size in the range of 3–15 nm.

5.4.7 Characterization of As-loaded Silver Released from the nAg-containing Gelatin Hydrogels

Prior to investigating the release characteristic of silver from the nAg-containing gelatin hydrogels that had been irradiated at 60 kGy, the actual amount of silver in these specimens needed to be determined. For this purpose, the specimens (i.e., 0.25, 1, 3, 5, 10% nAg-containing gelatin hydrogels) were dissolved in 50 mL of 50% nitric acid (HNO_3) solution. The obtained silver-containing solutions were then quantified for the actual content of as-loaded silver (by means of AAS) to be

65.65 ± 2.65%, 62.30 ± 1.33%, 61.06 ± 1.17%, 61.68 ± 0.66% and 61.53 ± 1.31% for the theoretical content of 0.25, 1, 3, 5 and 10% nAg-containing gelatin hydrogels, respectively. These values were used to arrive at the cumulative release of silver from the nAg-containing gelatin hydrogel specimens.

The release characteristic of Ag ions from 0.25, 1, 3, 5 and 10% nAg-containing gelatin hydrogel specimens that had been irradiated at 60 kGy was investigated by the total immersion method in PBS at the physiological temperature of 37 °C. The cumulative amount of silver, either in the form of Ag nanoparticles or residual free ions (Ag^+), that were released from these materials is reported in Figure 5.7 by the weight of Ag released divided by the actual Ag content (%) (see Figure 5.7a), the weight of Ag ions released (in mg) divided by the weight of the specimens (in g) (see Figure 5.7b) and the amount of Ag ions released (in ppm) divided by the weight of the specimens (in g) (see Figure 5.7c) as a function of the submersion time.

The release profiles of Ag ions from these gelatin hydrogels could be divided into 2 stages: the initial rather rapidly release within the first period of submersion and the quite slowly release afterwards. Especially, for the hydrogels that were prepared from the gelatin solutions containing higher $AgNO_3$ content (i.e., 3, 5, and 10%), this characteristic was dominant. The first period that the Ag ions released rapidly from the 3, 5, and 10% nAg-containing gelatin hydrogels was 6 h, 2 h, and 40 min, respectively, with the value of ~38.5, ~49.0, and 68.9%, respectively (see Figure 5.7a reported as the percentage of the weight of Ag ions released divided by the actual weight of the Ag ions presence in the specimens) while this period could not be seen clearly in the cumulative release profiles of Ag from the lower nAg-containing gelatin hydrogel specimens (i.e., 0.25% and 1%). The maximum amount of Ag ions released from the 0.25, 1, 3, 5, and 10% nAg-containing gelatin hydrogels on day 10 were ~4.8, ~9.7, ~57.3, 69.3, and ~90.8%, respectively. As expected, the maximum amount of Ag ions released from these hydrogels increased with increasing initial amount of nAg-containing in the gelatin solutions.

When reported either as the weight of Ag ions (in mg) or the amount of Ag ions (in ppm) released per gram of specimens, a similar trend of the released profiles of Ag ions from the corresponding hydrogels were also observed. The values of Ag ions released in the initial period from the 3, 5, and 10% nAg-containing

gelatin hydrogels at 6 h, 2 h, and 40 min, respectively, were ~6.2, ~14.4, and 38.5 mg/g, respectively (see Figure 5.7b reported as the weight of Ag ions released divided by the weight of the specimens). The total amount of Ag ions released from the 0.25, 1, 3, 5, and 10% nAg-containing gelatin hydrogels on day 10 were ~0.08, ~0.6, ~10.2, 20.4, and ~50.8 mg/g, respectively.

When reported as the amount of Ag ions (in ppm) released per gram of specimens, at the first stage of Ag ions released from the 3, 5, and 10% nAg-containing gelatin hydrogels at 6 h, 2 h, and 40 min, respectively, the cumulative amounts of silver released in to the media were ~248.2, ~576.1, ~1540.6 ppm/g, respectively (see Figure 5.7c reported as the amount of Ag ions released (in ppm) divided by the weight of the specimens). And these amounts finally increased to be ~3.2, ~24.0, 407.6, ~814.5, and ~2030.7 ppm/g for the 0.25, 1, 3, 5, and 10% nAg-containing gelatin hydrogels on day 10.

The release kinetics of Ag^+ from a carrier can be characterized according to the following equations (Peppas & Khare, 1993; Ritger & Peppas, 1987):

$$\frac{M_t}{M_\infty} = kt^n \quad \text{for} \quad \frac{M_t}{M_\infty} < 0.6 \tag{14}$$

where M_t is the cumulative amount of the Ag^+ released at an arbitrary time t, M_∞ is the cumulative amount of the Ag^+ released at an infinite time, n is an exponent characterizing the mechanism with which the release kinetics can be described, and k is the release rate of the Ag^+ that incorporate the physical character of the releasing system.

The case for normal Fickian diffusion is characterized by the value of n being 0.5 and Case II diffusion is by the value of n being 1.0, while the case for non-Fickian or anomalous diffusion is characterized by the value of n being between 0.5 an d 1.0 (Peppas &Khare, 1993; Ritger & Peppas, 1987). In case of the Fickian diffusion (i.e., n = 0.5), a straight line is expected when the fractional cumulative amount of Ag^+ released (i.e., M_t/M_∞) is plotted as a function of $t^{0.5}$ (Fig. 5.8a). The results from the analyses (i.e., values of parameters k and the r^2, which signifies the goodness of the fits) are summarized in Table 5.3. Based on the Fickian diffusion mechanism, the values of the rate parameter 'k' for all of the nAg-loaded gelatin

hydrogels were increase with increasing in the as-loaded silver in gelatin hydrogel. In case of the non-Fickian diffusion, the diffusion exponent 'n' and the rate parameter 'k' can be obtained from the values of the slope and the intercept of the plot of $\ln(M_t/M_\infty)$ versus ln(t)(Fig. 5.8b) and the results of such analyses are also summarized in Table 5.3. As expected, with increasing in the as-loaded silver in gelatin hydrogel, the value of 'k' was found to increase and the value are similar to the case of Fickian diffusion type of release mechanism at a given as-loaded Ag content.

5.4.8 Antibacterial Evaluation of nAg-containing Gelatin Hydrogels

The potential use of nAg-containing gelatin hydrogels as functional wound dressings was assessed by observing their antibacterial and bactericidal activity based on the disc diffusion method and the dynamic shakes flask test method, respectively against the model bacteria: *Staphylococcus aureus* and *MRSA*.

5.4.8.1 Disc Diffusion Method

Disc diffusion method was used to evaluate the antibacterial activity of nAg-containing gelatin hydrogels against *Staphylococcus aureus* and *MRSA* by measuring the inhibition zone length. The activity of the neat gelatin hydrogels and the drug pellets of vancomycin against these bacteria were used as a control. The length of inhibition zone around specimens reveals degree of sensibility of the microorganism. The strains susceptible to antibiotics exhibit larger inhibition zone length, whereas resistant strains exhibit smaller one (Ruparelia, J.P., *et. al*, 2008; Rujitanaroj, P., *et al,* 2008; Rattanaruengsrikul, V., *et al*, 2009).

Table 5.3 summarizes the average lengths of the inhibition zones (measured from the edge of the samples to the edge of the clear zones) for all of the samples investigated with the zone of inhibition of vancomycin disc (positive control) against *Staphylococcus aureus* and *MRSA* was 9.6 and 7.4 mm.

After 24 h of incubation at 37 °C, nutrient agar plates containing any types of nAg-containing gelatin hydrogels specimens (8 mm in diameter of circular disc with thickness about ~2.5-3 mm were cut from the as-irradiated hydrogels sheet) exhibited a larger zone of inhibition in the range of ~3.1-3.7 mm diameter against *Staphylococcus aureus over* against *to the MRSA* which their inhibition zones were in the range of ~2.4-3.2 mm. On the other hand, such

zones were discernible for the drug pellets about 9.59 mm against *Staphylococcus aureus* and 7.37 mm against MRSA. But, no inhibition zone against both types of bacteria was observed for the nutrient agar plates containing negative control of neat gelatin hydrogel specimens. The inhibition of bacterial growth around the specimens is due to the release of diffusible inhibitory compounds from nAg-containing gelatin hydrogels into the surrounding medium (Cooper, R., 2004; Kumar, R., *et al*, 2005; Bhattacharya, R., and Mukherjee, P., 2008; Rujitanaroj, P., *et al,* 2008; Singh, M., *et al*, 2008).

Clear trends of antibacterial activity of different concentrations of nAg-containing gelatin hydrogels that had been irradiated at various doses could not be seen from the observation clear zone results. Because many factors –such as the concentration of Ag-loaded, irradiation does that used for synthesize the hydrogels, the physical properties of as-prepared hydrogels and those of the as-synthesized Ag nanoparticles– have an effect on the diffusible of Ag ions from nAg-containing gelatin hydrogels through their bulk hydrogels into the bacterium-infested agar plates. It should be noted that swelling and weight loss properties and gel fractions of the as-prepared nAg-containing gelatin hydrogels, size of as-synthesized Ag nanoparticles has depended on the concentration of nAg-containing in the gelatin solution and the irradiation dose used for synthesize the nAg-containing gelatin hydrogels. Therefore, quantitative method for antibacterial evaluation was done by Dynamic shakes flask test method.

5.4.8.2 Dynamic Shakes Flask Test Method

Since there are many factors affect the observed clear zone around the nAg-containing gelatin hydrogels specimens, so an appropriate experiment of bactericidal activity of nAg-containing gelatin hydrogels were carried out via the dynamic shake flask test method according to the ASTM E 2149-01. This quantitative method provides the number of viable microorganisms in the suspension and the percent reduction after treated with antimicrobials agent.

The weighed neat and nAg-containing gelatin hydrogels that had been irradiated at 60 kGy are shake in a 50 ml of 10^5 CFU/mL concentrated either *Staphylococcus aureus* or *Methicillin-resistant Staphylococcus aureus* (MRSA) suspension for different contact times (i.e., 0, 20, 40, 60 min 2, and 3 h) at

37 °C. At specific contact times, 1 mL of the suspension was withdrawn and made serial dilutions with sterilized normal saline and spiral on plates (in triplicate) on nutrient agar. After 24 h of incubation at 37 °C, plates were observed for bacterial colony formation and calculated the percent reduction.

Table 5.4 and Table 5.5 (Figure 5.9) demonstrate effects of Ag nanoparticles content and contact times on the bactericidal efficacy of nAg-containing gelatin hydrogels that had been irradiated at 60 kGy against *Staphylococcus aureus* and *Methicillin-resistant Staphylococcus aureus* (MRSA), respectively.

At any contact times, it was observed that both percentage reduction of *Staphylococcus aureus* and *Methicillin-resistant Staphylococcus aureus* (MRSA) increased with increasing the content of Ag on the nAg-containing gelatin hydrogels. However, it was interesting to note that the optimum contact time to reach 99% reduction of either *Staphylococcus aureus* or *Methicillin-resistant Staphylococcus aureus* (MRSA) for all nAg-loaded gelatin hydrogels was different. For 0.25% nAg-loaded gelatin hydrogel irradiated at 60 kGy, it was observed that the contact time of 2 h was sufficient to obtain 99% reduction for *Staphylococcus aureus* whereas more than 3 h but less than 24 h was satisfactory to reach 99% reduction of the *Methicillin-resistant Staphylococcus aureus* (MRSA). However with increasing in the nAg content in the gelatin hydrogels, the sufficient time used to reach 99% reduction of viable bacterial was found to decrease. Apparently, the antibacterial efficiency of nAg-loaded gelatin hydrogel against *Methicillin-resistant Staphylococcus aureus* was lower than that against *Staphylococcus aureus*, most likely because MRSA was resistant to a number of different antibiotics, thus it was harder to treat than non-resistant bacteria.

5.4.9 Indirect Cytotoxicity Evaluation of Neat and nAg-containing Gelatin Hydrogels

The potential use of the nAg-containing gelatin hydrogels as antibacterial wound dressings was further assessed by investigating the cytotoxicity of these materials, using normal human dermal fibroblasts (NHDF) as reference. The cytotoxicity was evaluated indirectly by culturing NHDF with extraction media (prepared at the extraction ratio of 10 and 20 $mg \cdot mL^{-1}$) of the hydrogel specimens in

comparison with those that had been cultured with the fresh culture medium. The results are shown in Figure 5.10 for a given extraction ratio, the viabilities of NHDF for all of the nAg-containing gelatin hydrogel specimens were lower than that of the neat materials. The greater ratio of extraction media decreased the viabilities of the cells for all of the hydrogel specimens. Evidently, the cells that had been cultured with the extraction media from either the neat or the nAg-containing at any content that less than 3% in gelatin hydrogel specimens, at any given extraction ratio, exhibited the viability that was greater than 80% as compared to those of the cells that had been cultured with the fresh culture medium. The obtained results indicated that these materials released no substances in the levels that were harmful to the cells. On the other hand, the viabilities of cells that had been cultured with the 3% and 5% nAg-containing gelatin hydrogel specimens at any concentration of extraction ratio were lower than 80% with respect to that of the cells that had been cultured with the fresh culture medium. That means they are toxic toward skin cells.

5.5 Conclusion

Gelatin hydrogels containing Ag nanoparticles ($AgNO_3$ as a precursor at 0.25-10% base on the weight of gelatin powder) successfully prepared as antibacterial wound dressings by gamma irradiation. Irradiation of aqueous solution of gelatin containing Ag^+ ions resulted in the simultaneous cross-linking of gelatin; meanwhile Ag^+ ions undergo reduction, aggregation to form Ag nanoparticles within gelatin hydrogels. Eventually lead to the formation of gelatin–Ag nanocomposites. The formation of nAg was confirmed not only by the change in the color of the obtained hydrogels, but also by the observation of the surface Plasmon peak in the UV spectrum at around 420–460 nm. The gel fraction of nAg-containing gelatin hydrogels was higher when lower content of $AgNO_3$ were loaded and irradiated at higher doses were applied. The water retention and the weight loss of the neat gelatin hydrogels in three types of medium (i.e., distilled water, simulated body fluid; SBF and phosphate buffer solution; PBS) at any given immersion times decreased with increasing the irradiation dose. Both values were the highest when these hydrogels were submerged in the distilled water. Based on the water retention data in distilled

water, the cross-link density of the hydrogels was determined and it was found to increase with an increase in the dose used to irradiate the hydrogels and/or a decrease in the amount of $AgNO_3$ loaded into the gelatin solution. The total cumulative amount of silver released from the hydrogels was found to increase with an increase in the $AgNO_3$ content. The potential for use of the cross-linked nAg-containing gelatin hydrogels as wound dressings was assessed by antibacterial activity against Gram-positive *Staphylococcus aureus* and *MRSA* and indirect cytotoxicity against normal skin fibroblasts. The results showed that the hydrogels were effective against the two pathogens. The lower content of nAg-containing gelatin hydrogels needs longer time to achieve the 99% reduction of the initial amount of bacteria. These hydrogels appeared to be non toxic against the tested cells only in case of the low $AgNO_3$-loaded hydrogels (i.e., 0.25-1% Ag-loaded). Appropriate low content of nAg-containing gelatin hydrogels (i.e., 0.25-1%) could be used to be the effective antibacterial wound dressings with desirable properties.

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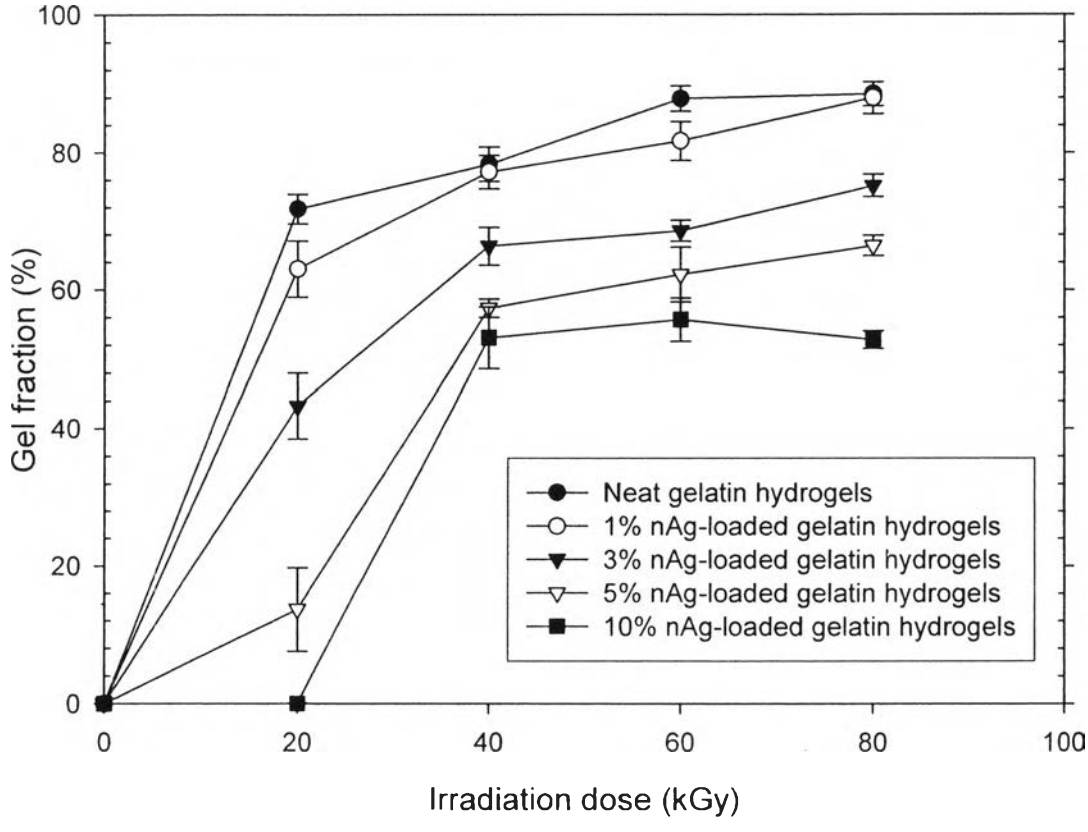
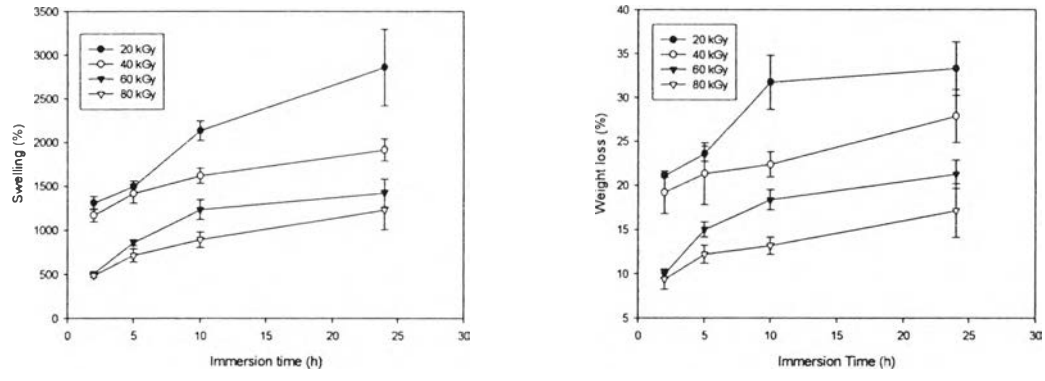
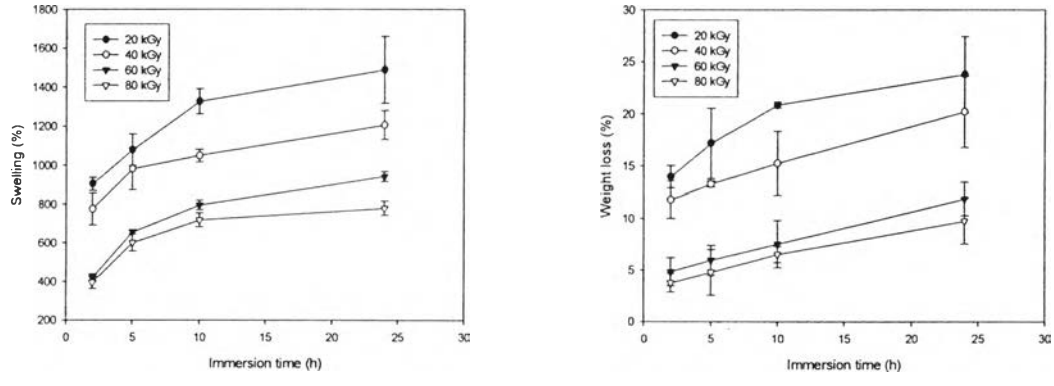


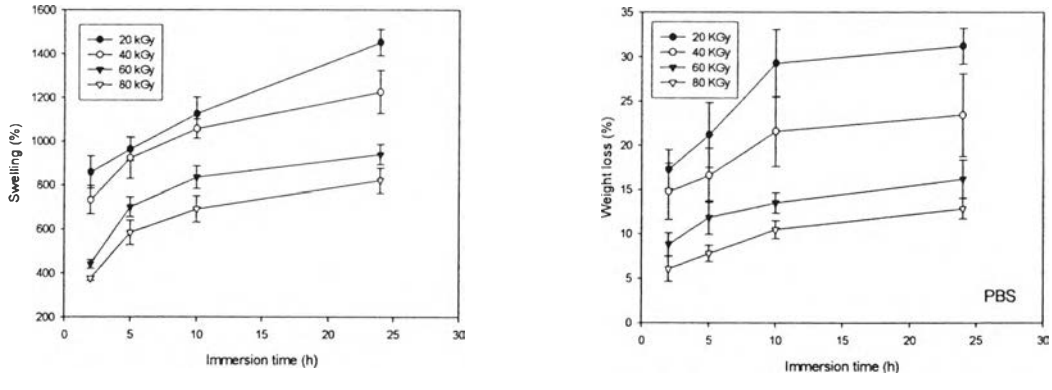
Figure 5.1 Gel fraction of neat gelatin and nAg-loaded gelatin hydrogels at various irradiation doses after having been extracted with 50 °C of distilled water at the violent shaking of 100 rpm (n=3).



(a)



(b)



(c)

Figure 5.2 Degree of swelling and weight loss of neat gelatin hydrogel specimens at various irradiation doses after submersion in (a) distilled water (pH 6.9), (b) simulated body fluid; SBF (pH 7.4) and (c) phosphate buffer solution; PBS (pH 7.4) as a function of time.

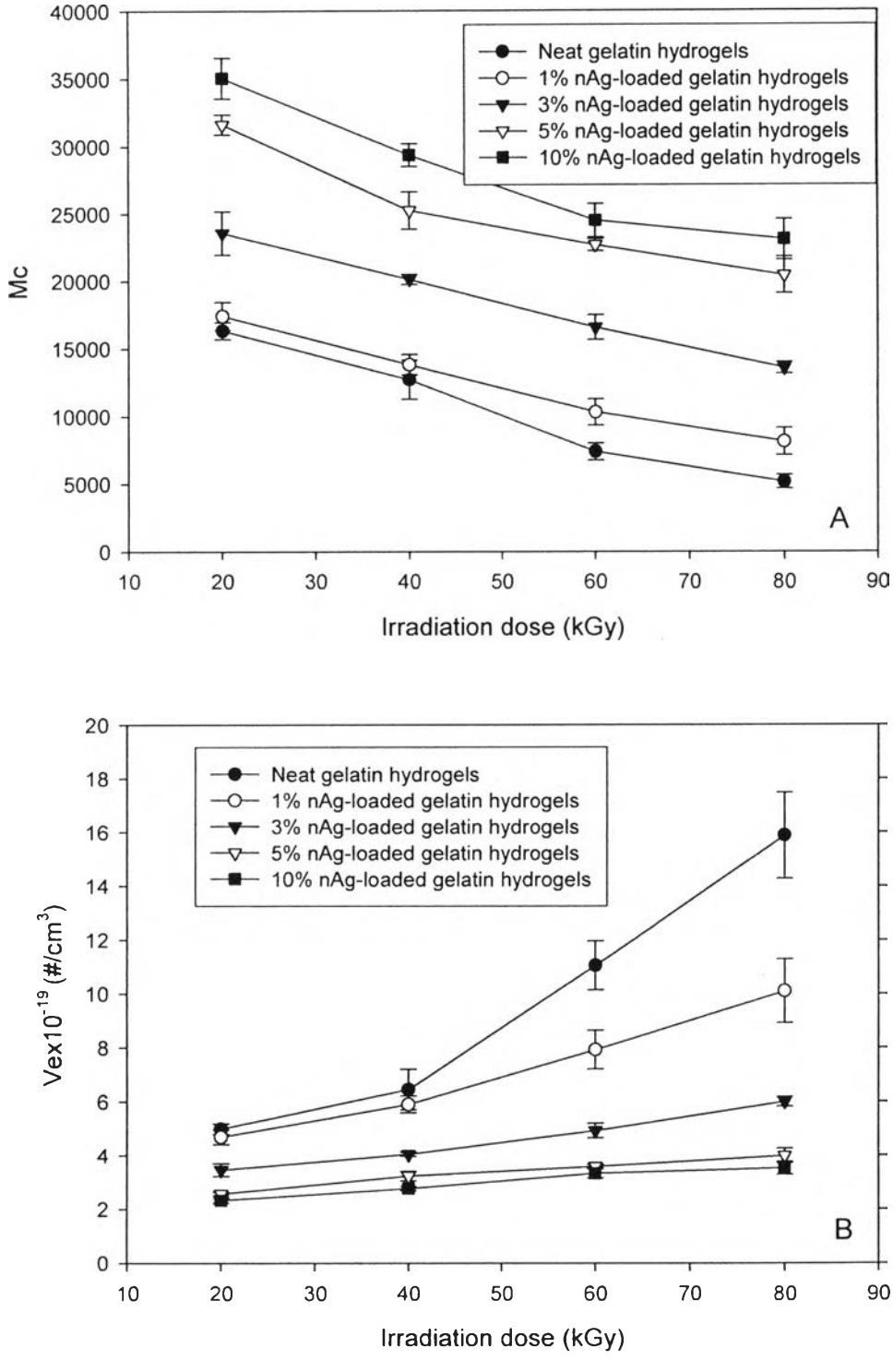


Figure 5.3 Number-average molecular weight of chain segments between cross-linking points (A) and cross-link density (B) of nAg-loaded gelatin hydrogels as a function of irradiation dose.

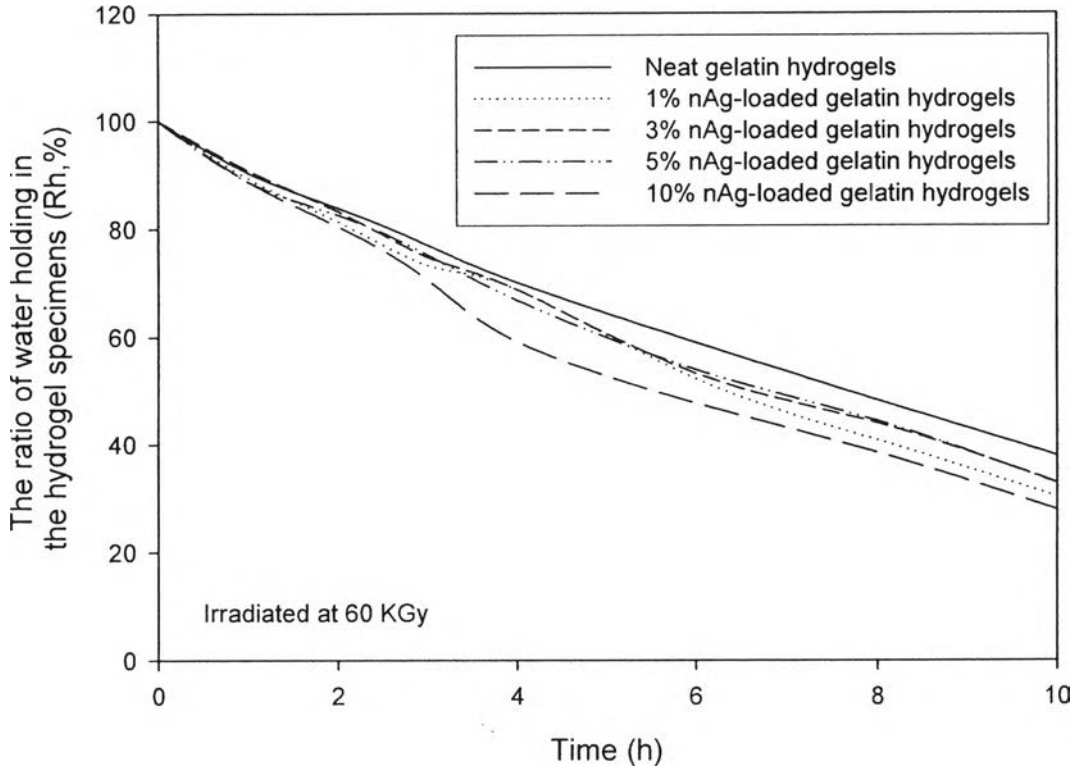


Figure 5.4 Ratio of water holding in the nAg-loaded gelatin hydrogel specimens that had been irradiated at 60 kGy as a function of time.

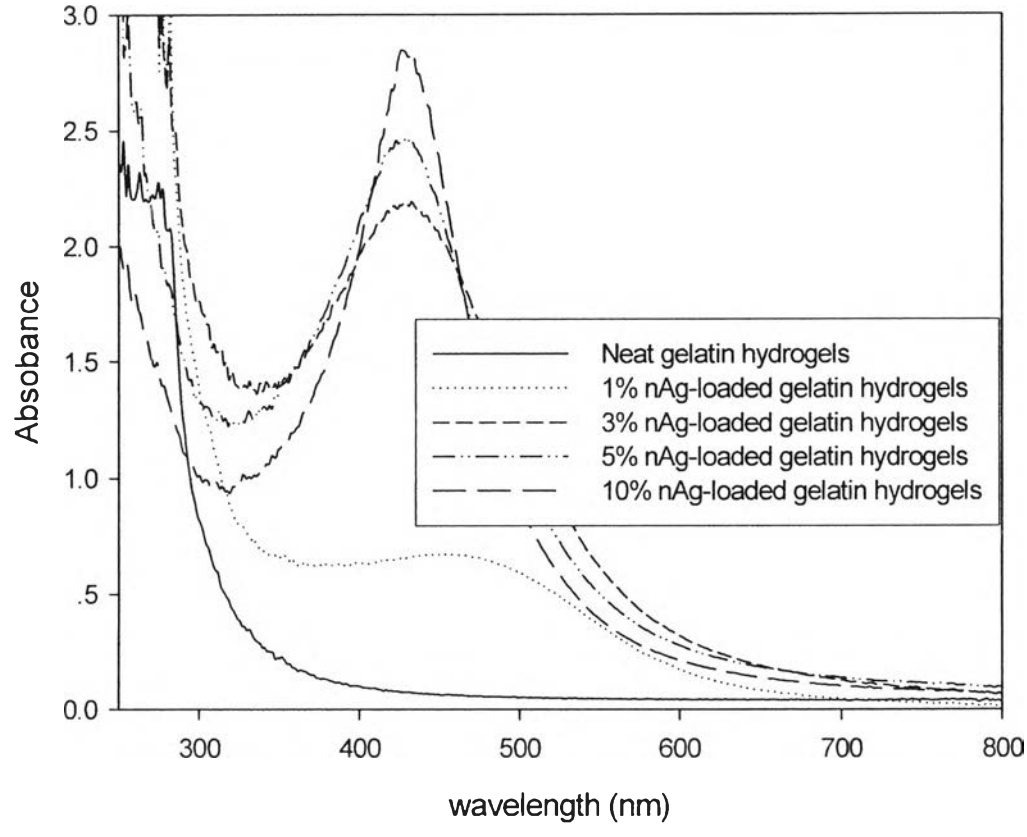
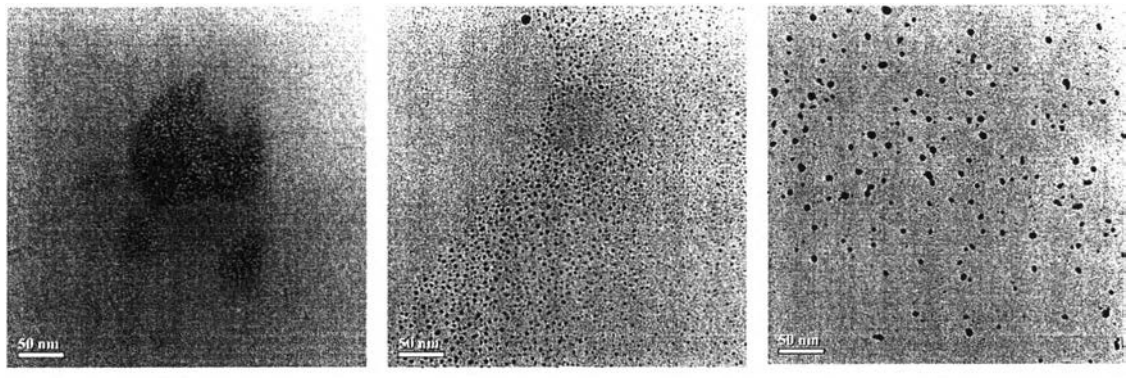
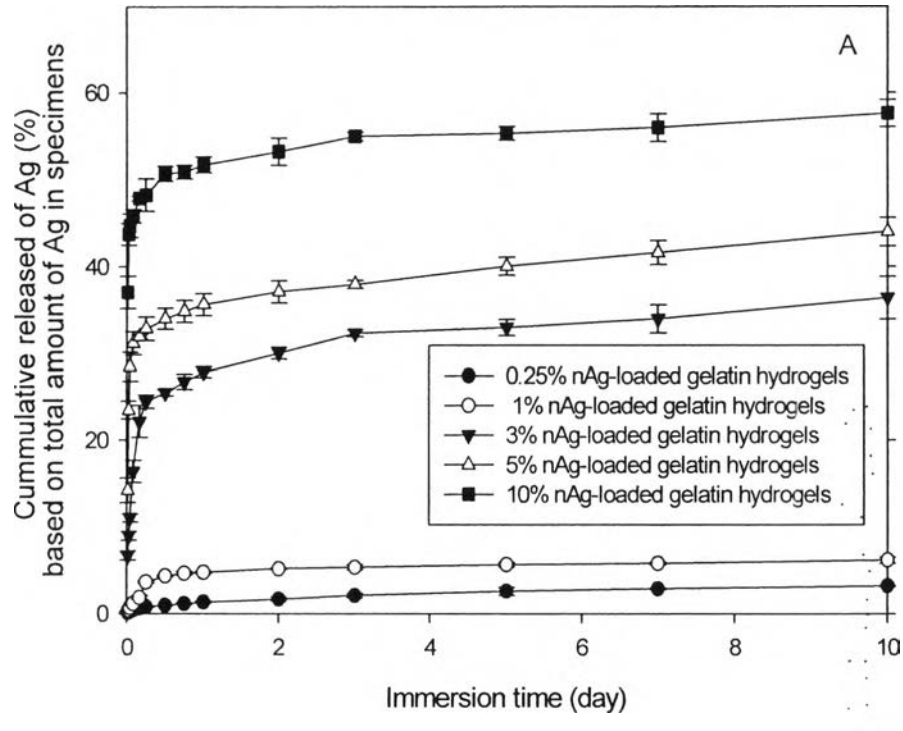


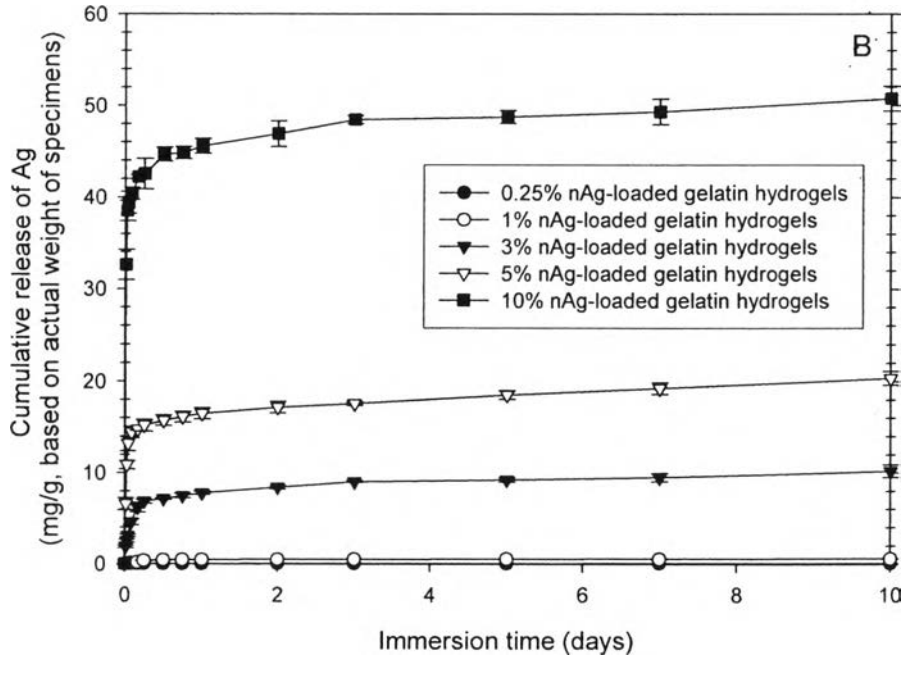
Figure 5.5 UV-vis absorption spectra of the base gelatin hydrogels and the Ag-loaded gelatin hydrogels that had been irradiated at 60 kGy at various contents of Ag-loaded.



(a) (b) (c)

Figure 5.6 Selected TEM images of nAg distributed in gelatin hydrogels (a) 3% nAg-loaded gelatin hydrogels after having been irradiated at 40 kGy, (b) 5% nAg-loaded gelatin hydrogels after having been irradiated at 40 kGy, (c) 10% nAg-loaded gelatin hydrogels after having been irradiated at 40 kGy.


A
Cummulative released of Ag (%)
based on total amount of Ag in specimens
0
20
40
60
0
2
4
6
8
10
Immersion time (day)
0.25% nAg-loaded gelatin hydrogels
1% nAg-loaded gelatin hydrogels
3% nAg-loaded gelatin hydrogels
5% nAg-loaded gelatin hydrogels
10% nAg-loaded gelatin hydrogels


B
Cumulative release of Ag
(mg/g, based on actual weight of specimens)
0
10
20
30
40
50
60
0
2
4
6
8
10
Immersion time (days)
0.25% nAg-loaded gelatin hydrogels
1% nAg-loaded gelatin hydrogels
3% nAg-loaded gelatin hydrogels
5% nAg-loaded gelatin hydrogels
10% nAg-loaded gelatin hydrogels

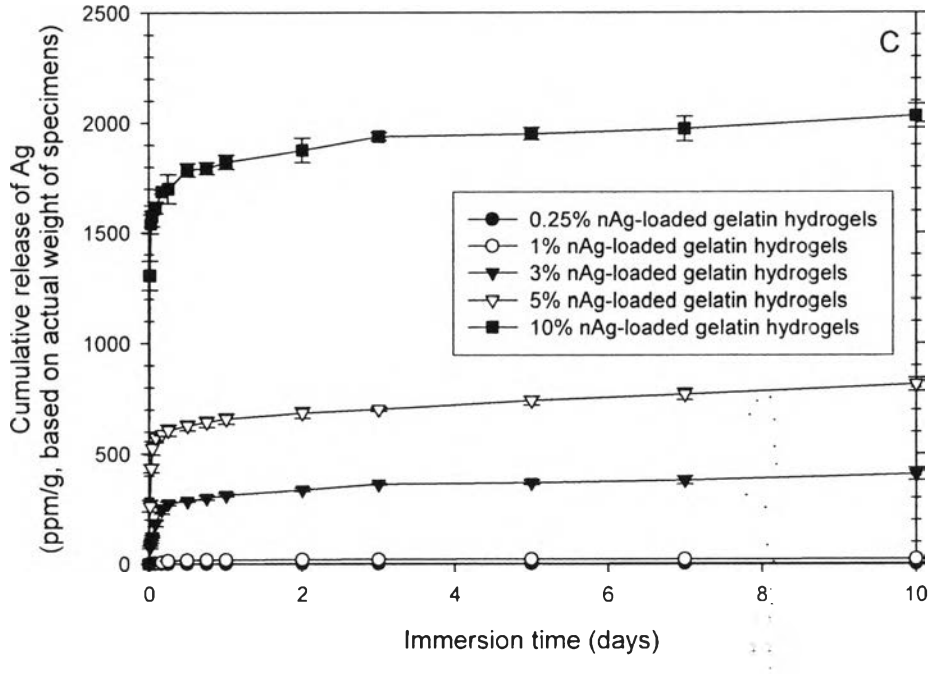
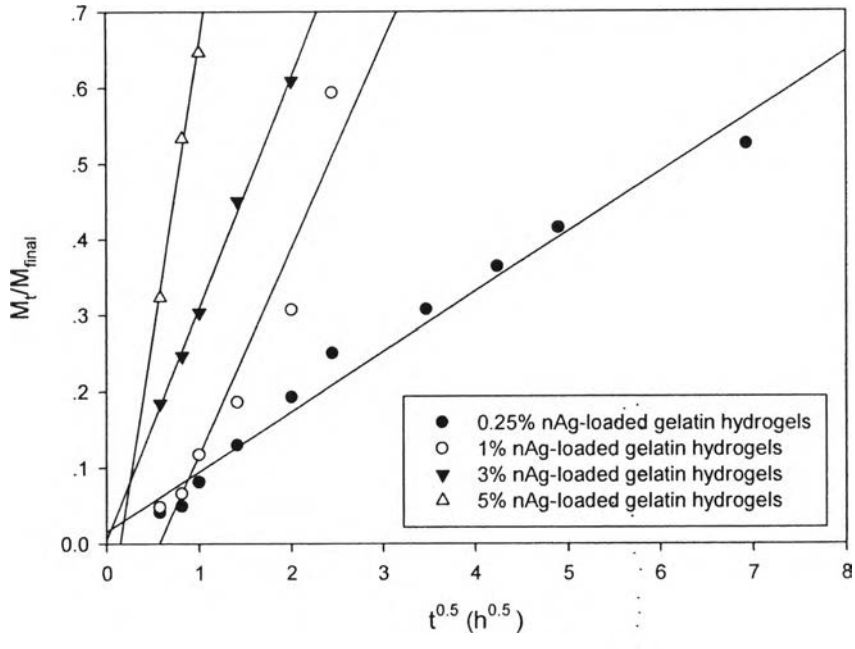
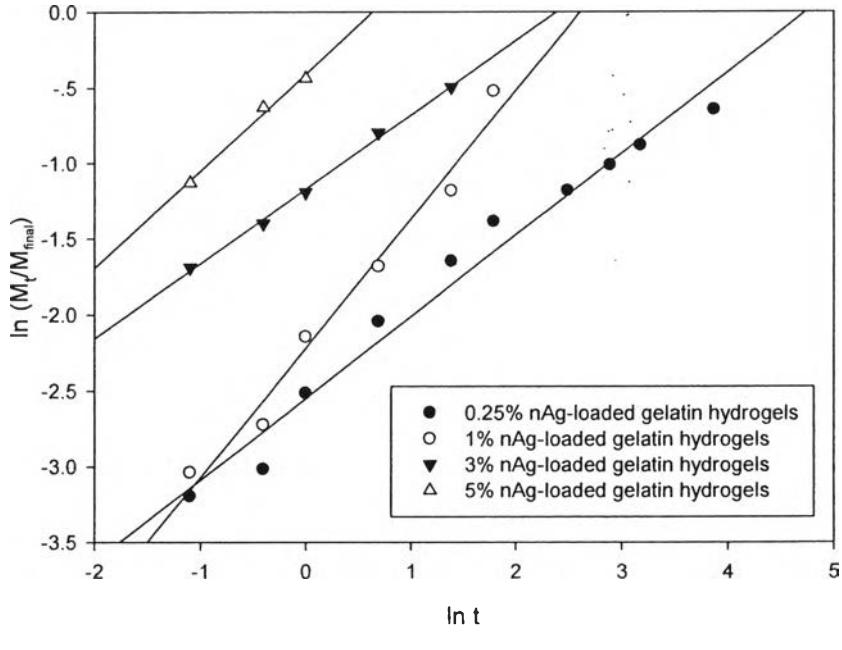


Figure 5.7 Cumulative release profiles of Ag from 0.25, 1, 3, 5, and 10% Ag-loaded gelatin hydrogels after having been irradiated at 60 kGy, reported as (A) the percentages of the released weights of Ag divided by the actual weights of Ag in the specimens and (B) the ratio of the released weights of Ag divided by the actual weights of the specimens (mg/g), and (C) the amount of the released Ag divided by the actual weights of the specimens (ppm/g) in the releasing medium of phosphate buffer solution (PBS) at the physiological temperature of 37 °C (n = 3).

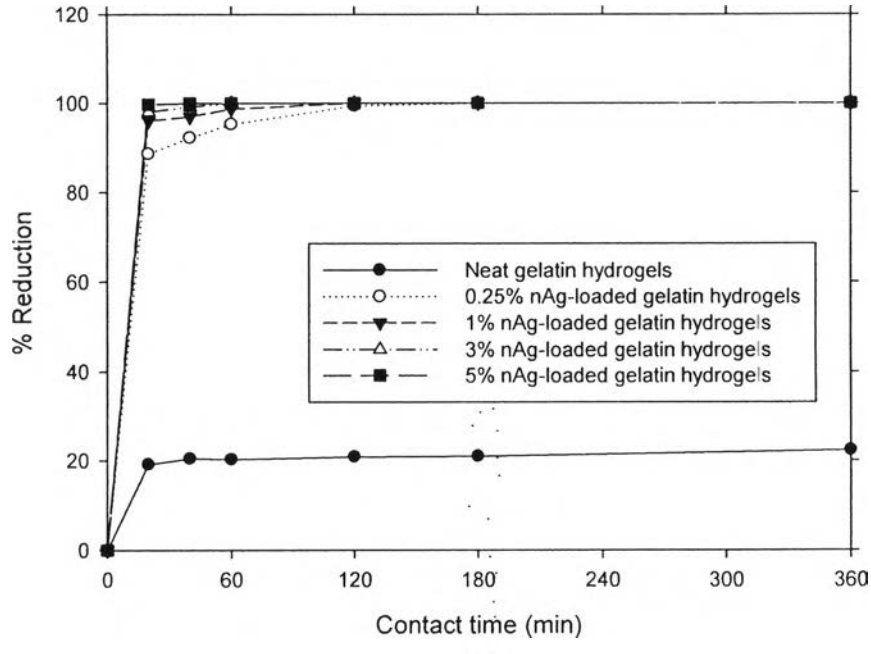


(a)

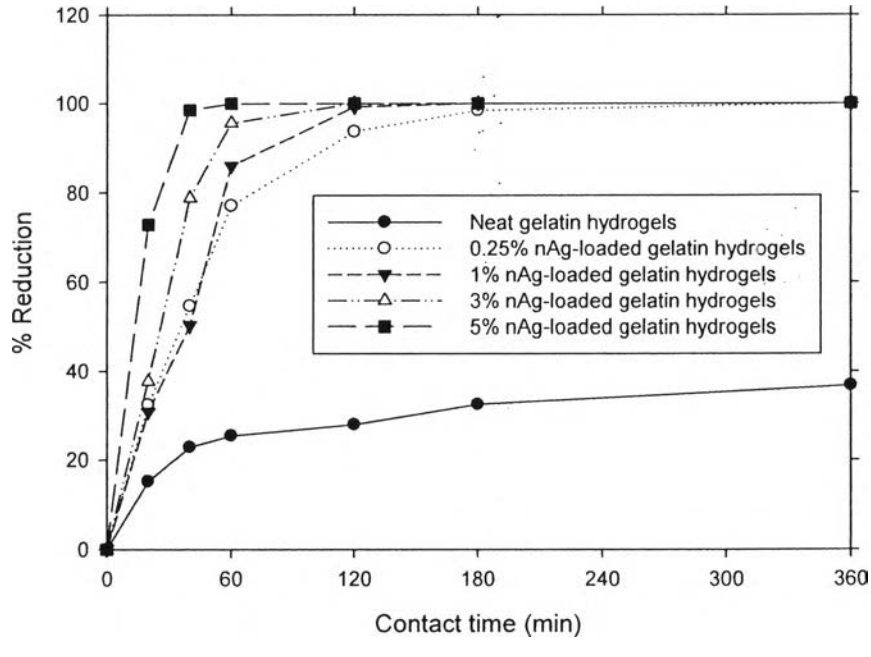


(b)

Figure 5.8 Fitting curves of the release kinetics of Ag^+ from nAg-loaded gelatin hydrogels irradiated with gamma ray at 60 kGy based on (a) Fickian diffusion type of release mechanism and (b) non-Fickian diffusion type of release mechanism.



(a)



(b)

Figure 5.9 Percent reduction of (a) *Staphylococcus aureus* and (b) *methicillin-resistant Staphylococcus aureus* after incubation with nAg-loaded gelatin hydrogel as a function of time.

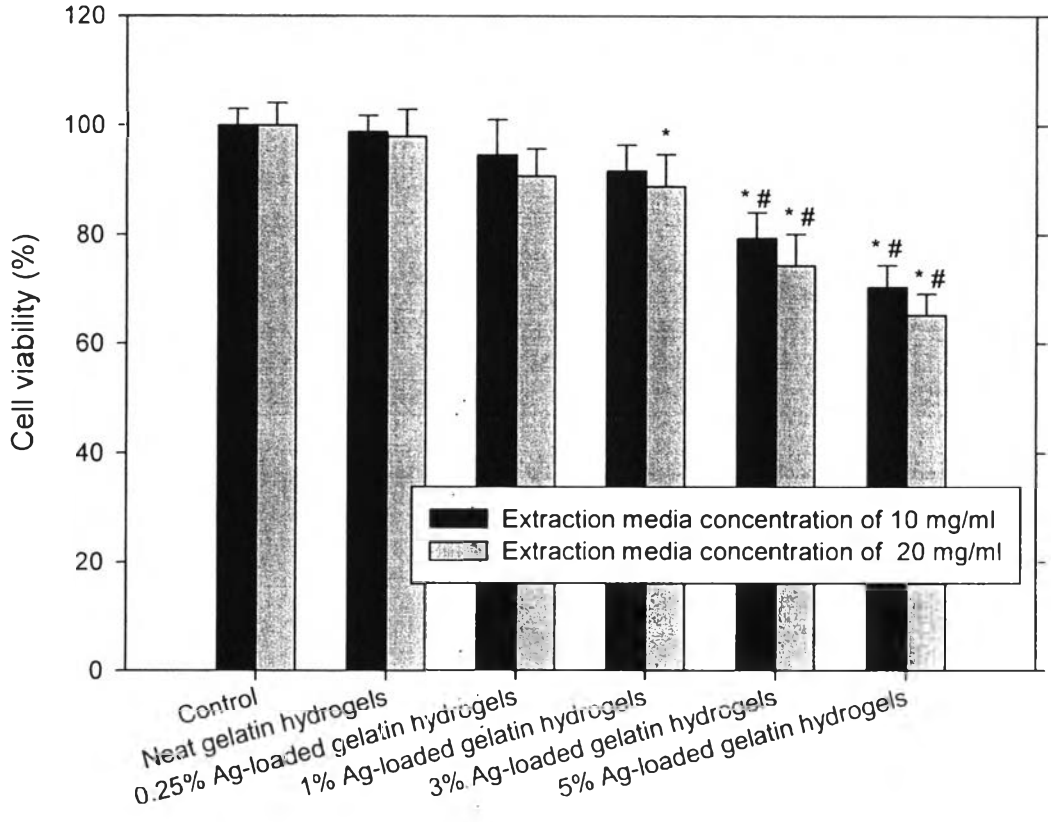


Figure 5.10 Viabilities of normal human dermal fibroblasts (NHDF) that were cultured for 24 h with extraction media concentration of 10 mg/ml and 20 mg/ml from neat and Ag-loaded gelatin hydrogels after having been irradiated at 60 kGy in comparison with viability of the cells that were cultured with fresh culture medium ($n = 3$).

*$p < 0.05$ compared with control at a given extraction media concentration.

Table 5.1 Water losing rate, $Rh_{,4h}$ and Water vapor transmission of 60 kGy-irradiated nAg-loaded gelatin hydrogels

Hydrogel irradiated at 60 kGy (thickness ~2.5 mm)	water losing rate (g/h)	$R_{h,4h}$ (%)	Water vapor transmission rate ($g/m^2/d$)
Neat gelatin hydrogels	6.9×10^{-2}	70.08 ± 0.97	2927
1% nAg-loaded gelatin hydrogels	7.3×10^{-2}	68.74 ± 2.43	2835
3% nAg-loaded gelatin hydrogels	7.5×10^{-2}	68.75 ± 1.21	2946
5% nAg-loaded gelatin hydrogels	7.5×10^{-2}	66.79 ± 1.51	2981
10% nAg-loaded gelatin hydrogels	7.7×10^{-2}	59.04 ± 2.59	3039

Table 5.2 Analyses on the release kinetics of Ag^+ from nAg-loaded gelatin hydrogels (n = 3)

Type of sample	Based on the Fickian diffusion type of release mechanism			Based on the non-Fickian diffusion type of release mechanism		
	k ($s^{-0.5}$)	n	r^2	k (s^{-n})	n	r^2
0.25% nAg-loaded gelatin hydrogels	0.0827	0.5	0.974	0.0781	0.5387	0.9753
1% nAg-loaded gelatin hydrogels	0.1860	0.5	0.9176	0.1088	0.7537	0.9728
3% nAg-loaded gelatin hydrogels	0.3082	0.5	0.9969	0.3098	0.492	0.9968
5% nAg-loaded gelatin hydrogels	0.6342	0.5	0.9585	0.6635	0.6409	0.9898

Table 5.3 Average zone lengths of the inhibition zones (measured from the edge of samples to the edge of the clear zones)

Irradiation dose (kGy)	Inhibition zone length (mm)							
	Staphylococcus aureus				*methicillin-resistant Staphylococcus aureus (MRSA)*			
	1% nAg	3% nAg	5% nAg	10% nAg	1% nAg	3% nAg	5% nAg	10% nAg
20	3.3±0.1	3.3±0.2	3.3±0.1	3.1±0.1	2.8±0.1	2.7±0.1	3.1±0.2	2.7±0.2
40	3.6±0.2	3.4±0.2	3.3±0.1	3.3±0.1	2.8±0.1	2.5±0.1	3.1±0.1	2.6±0.1
60	3.3±0.1	3.7±0.1	3.5±0.1	3.2±0.1	2.8±0.2	2.5±0.1	2.5±0.1	2.7±0.1
80	3.1±0.1	3.6±0.2	3.6±0.1	3.5±0.2	2.8±0.1	2.6±0.1	2.4±0.3	3.2±0.1

Note: the radius of specimen was 4 mm and the radius/the zone of inhibition of vancomycin disc (positive control) against *Staphylococcus aureus* and MRSA was 3/9.59 and 3/7.37 mm. No inhibition zone length around the neat gelatin hydrogels (negative control) against both *Staphylococcus aureus* and *MRSA* was observed.

Table 5.4 Percent reduction of *Staphylococcus aureus* after incubation with nAg-loaded gelatin hydrogel as a function of time

Staphylococcus aureus	% Reduction						
Contact time	0	20 min	40 min	1 h	2 h	3 h	6 h
Neat gelatin hydrogels	0	19.12	20.39	20.23	20.80	20.96	22.32
0.25% nAg loaded-gelatin hydrogels	0	88.58	92.21	95.25	99.35	99.96	99.96
1% nAg loaded-gelatin hydrogels	0	96.04	96.96	98.72	99.96	99.96	99.96
3% nAg loaded-gelatin hydrogels	0	98.03	99.16	99.96	99.96	99.96	99.96
5% nAg loaded-gelatin hydrogels	0	99.69	99.96	99.96	99.96	99.96	99.96

Table 5.5 Percent reduction of *methicillin-resistant Staphylococcus aureus* after incubated with nAg-loaded gelatin hydrogel as a function of time

methicillin-resistant Staphylococcus aureus	% Reduction					
Contact time (min)	20 min	40 min	1 h	2 h	3 h	6 h
Neat gelatin hydrogels	15.17	22.82	25.38	27.90	32.47	36.76
0.25% nAg loaded-gelatin hydrogels	32.36	54.62	77.07	93.62	98.38	99.93
1% nAg loaded-gelatin hydrogels	30.77	50.25	85.95	99.17	99.93	99.93
3% nAg loaded- gelatin hydrogels	37.67	78.80	95.52	99.93	99.93	99.93
5% nAg loaded- gelatin hydrogels	72.85	98.44	99.93	99.93	99.93	99.93