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Gamma Irradiation Synthesis and Characterization of AgNP/Gelatin/PVA Hydrogels for Antibacterial Wound Dressings

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ABSTRACT: An antibacterial hydrogel wound dressing was successfully synthesized by the gamma irradiation method. A gelatin solution was mixed with a poly(vinyl alcohol) (PVA) solution of similar concentrations at different weight ratios of 100 : 0, 80 : 20, and 60 : 40 w/w, and irradiated at 30, 40, or 50 kGy. The testing of physical properties showed that the addition of PVA could improve both durability and mechanical integrity. The 60 : 40 hydrogels irradiated at 30 kGy were optimal, and chosen to add silver nitrate at 0.25, 0.50, 0.75, or 1.00 wt % (based on the solid content) to improve the antibacterial properties. After gamma irradiation, silver nanoparticles (AgNPs) were formed. The AgNP/gelatin/PVA hydrogels were characterized for physical properties, cytotoxicity, and antibacterial activity. The AgNP/gelatin/PVA hydrogels could be used as antibacterial wound dressings because they exhibited appropriate physical properties, noncytotoxicity, and could inhibit the growth of tested bacteria. © 2014 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2014**, *131*, 41138.

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INTRODUCTION

Hydrogels are networks of hydrophilic polymers that are generated through crosslinking to form insoluble polymer structures. They can absorb water and aqueous fluids very well.[1,2] Hydrogels can be applied in many ways, especially in biomedical applications, such as contact lenses, drug delivery systems, and dressings for burn wounds.[3] Considering various merits, such as nontoxicity, nonadherence, ability to absorb exudates from wounds,[4] and ability to provide a moist environment good for wound healing, hydrogels have optimal properties to be used as wound dressings.[5]

The crosslinking methods used to create hydrogels can be divided into three main processes that include the physical, chemical, and radiation.[6,7] Hydrogels for biomedical applications can be crosslinked by the freeze-thawing technique[8] or using a glutaraldehyde aqueous solution.[9] These crosslinking methods can create hydrogel structures, but further sterilization of the hydrogels is mandatory. For this, the radiation crosslinking method is better than the other two methods. A dose of at least 25 kGy of gamma irradiation can sterilize the materials to levels that are recommended for medical products.[10]

Among the various materials that can be crosslinked to prepare a hydrogel, gelatin is versatile. Gelatin is derived from hydrolysis of collagen. Collagen is the important structural protein found in connective tissues and skin of vertebrates. Under certain conditions, gelatin can form transparent gels.[11,12] Advantages of gelatin are that it is easily obtainable, inexpensive, and biocompatible. However, structures made from gelatin, in their dry state, such as porous scaffolds show brittleness, less flexibility, and extremely fast degradation rate problems.[13–16] To solve this problem, a synthetic polymer was blended with a natural polymer to enhance the mechanical characteristics.[17] Poly(vinyl alcohol) (PVA), which is a water-soluble synthetic polymer, demonstrates biocompatibility, nontoxicity, and can also be crosslinked into hydrogels by gamma irradiation.[18] We have attempted to create a gelatin/PVA blend hydrogel.

Another issue of a hydrogel wound dressing is bacterial growth. Although hydrogels provide a wet environment that enhances wound healing, they also provide an optimal condition for the propagation of bacteria in the wound area.[19] The solution to this problem is the incorporation of an antibacterial agent into the hydrogels. Silver ions (Ag^+) have long been known as a

Additional Supporting Information may be found in the online version of this article.

broad-spectrum antibacterial agent. Apart from usage in wound care management,[20] they have been used to cure various diseases, for example, neonatal conjunctivitis.[21] Upon conversion into nanometallic form, such as silver nanoparticles (AgNPs), efficacy is increased. AgNPs act as reservoir of Ag^+ that can be supplied to the infected area surrounding of the wound. The mechanisms of Ag^+ are the key point of antibacterial activity. Ag^+ can interact with the thiol (sulfhydryl) groups and sulfur-containing proteins in the cell wall of bacteria, which can cause the protein to denature. This mechanism is vital to the antimicrobial activity of Ag^+.[22–24] They also can interrupt DNA replication.[25] Choi and Hu[26] found that specks of AgNPs, which were smaller than 5 nm, were related to the inhibition of the nitrifying bacteria. Moreover, Ag^+ can inhibit bacterial growth and amass in the vacuole and cell wall of bacteria.[27] Although the AgNPs and Ag^+ are harmful to bacteria, low concentrations are safe for human cells.[28] Sikareepaisan[29] evaluated the cytotoxicity of gelatin hydrogels with AgNPs and reported that the low silver nitrate ($AgNO_3$)-loaded hydrogels, with a $AgNO_3$ content of not more than 1 wt %, were not toxic to normal skin fibroblasts. Selvamurugan and coworkers[30] found that the biocomposite scaffold containing 50 m*M* $AgNO_3$ was nontoxic to osteoprogenitor cells of rat and osteosarcoma cell lines of human. AgNPs can be synthesized by many methods, such as citrate synthesis, borohydride, organic reducing agents, biosynthesis, and radiolytic methods.[31] The gamma radiation technique is one of the most suitable methods for producing AgNPs for medical uses, because there are no chemical agents or waste and it is an efficient sterilization process.

Several studies of hydrogels for wound dressings, which are prepared by gamma irradiation, have been reported in recent years.[32–36] Most of these research projects have focused only on physical or antibacterial properties of the hydrogels. Our study covered the essential properties of wound dressings, especially water vapor transmission rate (WVTR) which is an effective indicator of the moisture-retention capacity of wound dressings.[37,38] In this study, we have attempted to improve the mechanical properties of gelatin hydrogels by the incorporation of PVA. We varied the ratio of gelatin and PVA solutions, and the dose of gamma radiation. The physical properties were characterized to find which ratio and dose was optimal for use as hydrogel wound dressings. The AgNP/gelatin/PVA hydrogels were made by gamma radiation of $AgNO_3$ loaded in gelatin and PVA solution and tested for physical properties. The cytotoxicity test was carried out in order to evaluate *in vitro* toxicity to fibroblasts. The antibacterial activities of the hydrogels to gram-positive and gram-negative bacteria were also investigated.

EXPERIMENTAL

Materials

Gelatin (type A; porcine skin; 170–190 g Bloom) was purchased from Fluka (Switzerland). PVA with a hydrolysis degree of 99.0–99.8% (molecular weight = 89,000–98,000 Da) was purchased from Sigma-Aldrich (USA). $AgNO_3$ was purchased from Fisher Scientific (UK). All of the polymers and chemicals were used at analytical reagent grade without further purification.

Preparation

Preparation of Neat Gelatin Hydrogels. Gelatin powder was dissolved in distilled water at 45°C and stirred at 300 rpm for 40 min to make a 15 wt % solution. The gelatin solution was packed into a nylon bag. The samples were irradiated with ^{60}CO gamma-rays at Synergy Health (Thailand) at dose rates of 5 kGy/h. Gamma radiation dosages were targeted at 30, 40, and 50 kGy.

Preparation of Gelatin/PVA Hydrogels. The gelatin solution was prepared as described above. PVA solution was prepared by dissolving PVA powder in distilled water at 95°C and stirred at 300 rpm for 40 min to make a 15 wt % solution. The gelatin solution was poured into the PVA solution to make gelatin with PVA ratios of 80 : 20 and 60 : 40 w/w. The solution was blended at 80°C, and stirred at 300 rpm for 30 min. Finally, the solution was packed and irradiated as described above.

Preparation of AgNP/Gelatin/PVA Hydrogels. A gelatin/PVA solution was prepared as described above. $AgNO_3$ at 0.25, 0.50, 0.75, or 1.00 wt % (base on solid content) was added to the gelatin/PVA solution and stirred at 300 rpm for 30 min. The solution was packed and irradiated at 30 kGy. To eliminate air bubbles, all solutions were vibrated with an ultrasonic vibration machine for 15 min before being packed into a nylon bag.

Characterization of the Hydrogels

Water Holding Capacity and Swelling Behavior. The hydrogels were cut into 1.5 cm diameter discs and immersed in 50 mL of phosphate buffered saline (PBS) at 37°C, and shaken at 50 rpm for 24 h. Next, the hydrogels were removed from the medium. The solution on the surface of the hydrogels was absorbed by tissue paper. The swelled hydrogels were dried in an oven at 70°C for 48 h to a constant weight. The water holding capacity and swelling behavior of the hydrogels were evaluated by the following formulas:

$$\text{Water holding capacity } (\%) = (W_s - W_d)/W_s \times 100, \quad (1)$$

$$\text{Swelling behavior } (\%) = (W_s - W_i)/W_i \times 100, \quad (2)$$

where W_s is the weight of swollen gel, W_d is the weight of dried gel, and W_i is the weight of the initial sample (before being immersed in PBS).

Water Vapor Transmission Rate. WVTR of the hydrogels was measured as specify by ASTM standard E96-00[39] with some modifications. Distilled water (10 mL) was added to glass cups. The hydrogels were cut into 3.5 cm diameter discs and each was placed on the mouth of the cups. The hydrogels were fastened by binding with a paraffin film along the edges of the cups. The cups with the hydrogels were incubated at 37°C and 35% relative humidity, with the conditions maintained by using saturated solutions of magnesium chloride. The component was weighted at regular intervals. Weight loss and time were used to determine slope by function in Excel (Excel 2007; Microsoft). Slope represented weight loss per hour on average. To calculate WVTR in g/m^2/day, slope was multiplied by 24. WVTR was estimated by the following formula:

$$\text{WVTR} = (\text{slope} \times 24)/\text{Area of hydrogel } (\text{m}^2). \quad (3)$$

Tensile Test. The samples of each hydrogel were cut to a dumbbell shape according to the ASTM standard D638-03[40] with some modifications ($n = 5$). The dumbbell shape had a total length of 115 mm, a total width of 25 mm, and a width of narrow section of 10 mm. The tensile test was completed by a

universal testing machine (Lloyd, model LRX) at a speed of 22 mm/min with a preload of 0.01*N*.

***In Vitro* Biodegradation.** *In vitro* biodegradation of the hydrogels was studied by incubating in an enzymatic solution. The hydrogels were cut into cubes (100 mg). The media that imitated biodegradation was PBS with 1×10^4 U/mL of lysozyme concentration.[41] The incubation times were 6, 12, 18, and 24 h at 37°C. At the appointed time, the remaining hydrogels were washed to remove the residual enzyme and then freeze-dried. The percentage of weight remaining after degradation of the dried hydrogels was calculated.

Gel Fraction

The hydrogels were cut into 1.5 cm diameter discs and incubated in an oven at 50°C for 24 h. The dried hydrogels were then immersed in 50 mL of distilled water (50°C) for 2 h and dried in the oven at 50°C again until they dried completely. A gel fraction was calculated with the following formula:

$$\text{Gel fraction } (\%) = (W_d/W_i)\times 100, \quad (4)$$

where W_d is the weight of the dry gel after extraction and W_i is the initial weight of the dry gel before extraction.

Scanning Electron Microscope (SEM). The morphology of the freeze-dried AgNP/gelatin/PVA hydrogels was observed using SEM (Hitachi/S-4800; Japan). SemAfore program (JEOL SemAfore Software, Germany) was used to measure pore size and maximize histogram of the SEM images.

Fourier Transform Infrared (FTIR) Spectroscopy. The structure of the hydrogels was determined by FTIR spectroscopy (Perkin Elmer, Spectrum One, USA) in the wave number range of 4000–515 cm^{-1}.

Characterization of AgNP Formation

Ultraviolet–Visible (UV–vis) Analysis. The formation of AgNPs in the hydrogels was determined by using a Shimadzu UV-2550 UV–vis spectrophotometer (USA), which monitored the appearance of the surface plasmon resonance (SPR) band.

Transmission Electron Microscope (TEM). The AgNP/gelatin/PVA hydrogels were cut into small pieces and freezed at −40°C for 24 h, and then dried in a lyophilization machine (Labconco, USA) for another 24 h. The tiny freeze-dried hydrogels were ground and dissolved into 1 mL of ethanol for 30 min. Then 10 μL of solution was dropped on a copper grid and left to air-dry. The grids were examined by a JEOL JEM-2100 TEM (Japan) to image the AgNPs in the hydrogels. SemAfore program (JEOL SemAfore Software, Germany) was used to measure the size of AgNPs and maximize histogram of some images.

Energy-Dispersive X-ray Spectroscopy (EDX). The freeze-dried AgNP/gelatin/PVA hydrogels were analyzed by EDX (Hitachi/S-4800; Japan) to confirm that the particles in the hydrogels were silver particles.

Silver-Release Assay. The release characteristics of silver from the AgNP/gelatin/PVA hydrogels were investigated by diffusion through a cellulose acetate (CA) membrane. The hydrogels (discs of 1.5 cm in diameter) were placed on the CA membrane (diameter 2.5 cm, pore size 0.45 μm; vertical) on the PBS medium in the cell body of a Franz cell. The temperature of the system was 37°C at a specific diffusion time ranging between 1 and 24 h. The amount of silver in the PBS solution was determined by an atomic absorption spectrophotometer (Varian model AA280FS). It should be noted that a new sample set was used for each time point.

Cytotoxicity and Antibacterial Testing

Indirect Cytotoxicity Testing. Indirect cytotoxicity testing was adapted from the ISO10993-5 standard test method.[42] First, normal human dermal fibroblasts (NHDF; 11th–15th passage) were cultured in a 10,000 cell·well^{-1} tissue-culture plate in serum-containing Dulbecco's modified Eagle's medium for 24 h to allow cell attachment. Second, NHDF were then starved with serum-free medium (SFM) for 24 h. During the preparation of NHDF, the gelatin/PVA hydrogels and AgNP/gelatin/PVA hydrogels were cut into small pieces and immersed in SFM at an extraction ratio of 10 mg·mL^{-1} for 24 and 72 h. Next, the medium in the wells with NHDF was replaced with an extraction media, and cells were incubated for 24 h. Finally, MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was used to determine the viability of the cells at absorbance ([A]) at a wavelength of 550 nm, which was measured by a microplate reader. Cell viability was evaluated by the following formula:

$$\text{Cell viability } (\%) = ([A] \text{ test}/[A] \text{ control})\times 100. \quad (5)$$

Antibacterial Testing. The antibacterial activity of the hydrogels was tested against both gram positive and gram negative bacteria. Bacteria in this testing were *Staphylococcus aureus* (*S. aureus*; gram-positive; ATCC 25023), methicillin-resistant *S. aureus* (MRSA; gram-positive; ATCC 43300 was kindly provided from Microbiology Laboratory, Pramongkutkao Hospital), and *Escherichia coli* (*E. coli*; gram-negative; ATCC 25922). The gelatin/PVA hydrogels without AgNPs were used as the control group.

Antimicrobial Activity Assays. Quantitative analysis of the antimicrobial activity of the the AgNP/gelatin/PVA hydrogels was adapted from the work of Theapsak et al.[43] Briefly, colonies of bacteria were put into broth solutions, which were prepared by mixing nutrient broth (NB), pH 6.9, without NaCl (Sisco Research Laboratories; India) with 100 mL of sterile deionized water. The solution was cultured in a shaking incubator at a speed of 150 rpm and 37°C for 24 h. The bacterial suspensions were attenuated with 0.85% sterile NaCl aqueous solution. The dilution factor of *E. coli*, *S. aureus*, and MRSA were 10^5, 10^6, and 10^3, respectively. The hydrogels (discs of 1.5 cm in diameter; ~0.65 g) were loaded into the bacterial suspensions and incubated for 3 h in a shaking incubator (37°C, 150 rpm). Then, 100 μL of the suspension was spread on a NB agar plate (in triplicate). The plates were incubated at 37°C for 24 h. Then the bacteria colonies on the agar plate were counted. The antibacterial efficacy (ABE in %) of the AgNP/gelatin/PVA hydrogels was calculated based on the following equation:

$$\text{ABE } (\%) = [(V_c - V_t)/V_c]\times 100, \quad (6)$$

where V_c is the number of viable bacterial colonies of the control and V_t is the number of viable bacterial colonies of the AgNP/gelatin/PVA hydrogels.

Zone of Inhibition Test. The evaluation of antibacterial activity of the AgNP/gelatin/PVA hydrogels followed the US Clinical and Laboratory Standards Institute disc diffusion method and 30 μg vancomycin drug disc and 10 μg gentamicin drug disc (BD BBL™ Sensi-Disc™, USA) were used as control antibacterial drugs for gram-positive bacteria and gram-negative bacteria, respectively. The hydrogel discs of 1.5 cm in diameter and drug discs were placed on NB agar in a Petri dish and incubated at 37°C for 24 h. The clear zones, which are the area has no growth of bacteria, were measured from the edge of the disc.

Statistical Analysis

Data were presented as means ± standard deviation (SD). A t-test was used for statistical analysis (Data Analysis; Excel 2007; Microsoft). The statistical significance was accepted at 0.05 confidence level ($P < 0.05$).

RESULTS AND DISCUSSION

Preparation of Hydrogels

Hydrogels were successfully synthesized as jelly-like sheets. The 100 : 0 or neat gelatin hydrogels were transparent pale yellow, while the gelatin/PVA hydrogels were slightly opaque. The brown color of the hydrogels with AgNPs was unlike other hydrogels. The hydrogels performed good liquid absorption. After 4 h of PBS immersion at 37°C and shaken at 50 rpm, the diameter of hydrogels enlarged from 1.5 to 2.0 cm. Photographs of hydrogels are shown in Supporting Information Figure S1.

Our research attempted to create a practical wound dressing in the form of a hydrogel sheet, which was crosslinked by the irradiation method. The main components of the hydrogel were gelatin and PVA. The radiation crosslinking reaction of gelatin began with the hydroxyl radicals ($^{\bullet}OH$).[44] The hydrogen atoms from gelatin were withdrawn by the hydroxyl radicals and then carbon-centered radicals in the gelatin chain were produced [eq. (7)]. Gel and Gel$^{\bullet}$ represent gelatin and gelatin macroradical, respectively. The reactions of the gelatin macroradicals occurred continuously and the gelatin was crosslinked by this phenomenon [eqs. (8) and (9)].

$$^{\bullet}OH + Gel \rightarrow Gel^{\bullet} + H_2O \quad (7)$$

$$Gel^{\bullet} + Gel \rightarrow Gel - Gel^{\bullet} \quad (8)$$

$$Gel - Gel^{\bullet} + Gel - Gel^{\bullet} \rightarrow \text{Crosslinked gelatin} \quad (9)$$

Another biomaterial that was used was PVA. The radiation crosslinking reaction of PVA also started with hydroxyl radicals.[45] The reactions were represented by eqs. (10) and (11).

$$^{\bullet}OH + PVA\,(H) \rightarrow PVA^{\bullet} + H_2O \quad (10)$$

$$PVA^{\bullet} + PVA^{\bullet} \rightarrow PVA - PVA \text{ Crosslinked PVA} \quad (11)$$

The gelatin reacted with the hydroxyl group of PVA to form crosslinked bonds between gelatin and PVA.[46] The reaction was represented by eq. (12).

$$PVA^{\bullet} + Gel^{\bullet} \rightarrow PVA - Gel \text{ Crosslinked gelatin with PVA} \quad (12)$$

These free radical polymerizations formed the crosslinked hydrogels. The initial part of this research was to explore the most appropriate ratio of gelatin and PVA and dose of radiation to create a hydrogel sheet to be used as a wound dressing. Therefore, we varied the proportions of gelatin solution and PVA solution into ratios of 80 : 20, and 60 : 40 w/w. The proportion of gelatin was more than 50% of total weight because the main ingredient was gelatin, which is a natural polymer. The lowest radiation dose was 30 kGy, which was higher than the minimum sterilization dose of 25 kGy.[10] After we created the gelatin/PVA hydrogels at various ingredient and radiation doses, the hydrogels were tested to compare wound dressing properties. The most appropriate hydrogel to use as a wound dressing was found to be 60 : 40 w/w at a 30 kGy irradiation dose. We selected these hydrogels to create the AgNP/Gelatin/PVA hydrogels.

The AgNPs in this study were *in situ* synthesized by gamma irradiated induction of silver salt ($AgNO_3$). When the water in the solution was irradiated, hydrated electrons (e^-_{aq}) and hydrogen free radicals ($H^{\cdot}$) were produced from water radiolysis. The hydrated electrons reduced Ag^+, which originated from the dissolution of $AgNO_3$ in water. This occurrence created the neutral Ag^0 atoms.[47–50] The reactions were represented by eqs. (13) and (14). The aggregation of the neutral Ag^0 atoms occured continuously and finally leads to the formation of AgNP.[50–52] The reactions were represented by eqs. (15)–(17).

$$H_2O \rightarrow OH^{\bullet}, e^-_{aq}, H^{\bullet}, H_2O_2, H_2, H^+, \ldots \quad (13)$$

$$Ag^+ + e^-_{aq} \rightarrow Ag^0 \quad (14)$$

$$Ag^0 + Ag^+ \rightarrow Ag_2{}^+ \quad (15)$$

$$Ag_2{}^+ + Ag^+ \rightarrow Ag_3{}^+ \quad (16)$$

$$Ag_n{}^+ + Ag^+ \rightarrow Ag^+_{n+1} \quad (17)$$

Besides, the polymeric radicals PVA$^{\bullet}$, amino (—NH_2), and hydroxyl (—OH) groups of the gelatin reacted with Ag^+ to form silver particles.[36,53,54] Gamma irradiation has been accepted as the useful approach to create AgNP/hydrogel nanocomposites within a single step. The irradiation technique is clean and safe to the environment because there are no crosslinking/reducing chemical substances or waste/byproducts. The results of the testing of the hydrogels with and without AgNPs were presented and discussed together.

Characterizations of Hydrogels

Water Holding Capacity and Swelling Behavior. The outstanding property of hydrogels for wound dressings is its water holding capacity. Hydrogels can maintain a moist wound environment, which is a desirable characteristic of a wound dressing.[55] PBS was selected to use as a solution in water holding capacity testing. Kim et al.[56] suggested that polymers for wound dressing applications should have a water holding capacity of 90–96%, which can prevent gathering of exudates in the wound area and absorb water very well. As shown in Table I, all of the hydrogels show similar water holding capacities, which is ~96%, close to Kim's suggestion.

The gelatin and gelatin/PVA hydrogels in this study showed good swell behavior since gelatin and PVA are hydrophilic polymers. Gelatin is composed of 19 types of amino acids. The polar amino acids in gelatin, which are methionine, tyrosine, and cystine, favorable contact with water to make gelatin hydrophilic.[57] PVA is a fine hydrophile polymer because of the

Table I. Water Holding Capacity, Swelling Behavior, Gel Fraction, Stress at Maximum Load, and Percentage Strain at Maximum Load of Hydrogels

Hydrogels gelatin : PVA (kGy)	Water holding capacity (%)	Swelling behavior (%)	Gel fraction (%)	Stress at maximum load (N/mm^2)	Percentage strain at maximum load (%)
100 : 0 (30)	$96.64 \pm 0.25^{a,b}$	$196.80 \pm 27.15^{a,b}$	78.03 ± 1.39	$0.0439 \pm 0.0037^{a,b}$	$97.00 \pm 20.14^{a,b}$
80 : 20 (30)	96.69 ± 0.44^{c}	180.82 ± 45.78	71.24 ± 4.74^{a}	0.0403 ± 0.0078	$141.32 \pm 28.29^{*}$
60 : 40 (30)	$95.46 \pm 0.65^{*,d}$	171.00 ± 31.41^{c}	$79.39 \pm 0.36^{b,c}$	0.0402 ± 0.0059^{c}	$159.98 \pm 12.88^{*,c}$
100 : 0 (40)	95.51 ± 0.03^{a}	$139.82 \pm 3.17^{b,d}$	76.02 ± 2.21	$0.0319 \pm 0.0023^{a,d}$	58.64 ± 11.21^{a}
80 : 20 (40)	95.79 ± 0.90	138.92 ± 10.52	73.87 ± 4.07^{d}	0.0329 ± 0.0083	$128.25 \pm 25.00^{*}$
60 : 40 (40)	$94.71 \pm 0.47^{*}$	128.94 ± 12.93	$84.47 \pm 0.88^{*,c}$	0.0305 ± 0.0033^{c}	$140.11 \pm 22.66^{*}$
100 : 0 (50)	95.07 ± 0.40^{b}	$123.77 \pm 3.61^{a,d}$	77.48 ± 0.94	$0.0281 \pm 0.0016^{b,d}$	50.47 ± 11.09^{b}
80 : 20 (50)	94.34 ± 0.44^{c}	119.05 ± 14.48	$80.97 \pm 0.19^{*,a,d}$	$0.0362 \pm 0.0044^{*}$	$113.22 \pm 16.17^{*}$
60 : 40 (50)	$94.12 \pm 0.24^{*,d}$	117.51 ± 3.65^{c}	$89.27 \pm 3.48^{*,b}$	0.0330 ± 0.0051	$123.69 \pm 28.30^{*,c}$
60 : 40 (30) $AgNO_3$ 0.25 wt %	95.86 ± 0.16	$226.74 \pm 0.96^{\#}$	77.52 ± 1.92	$0.0256 \pm 0.0075^{\#}$	179.56 ± 15.79
60 : 40 (30) $AgNO_3$ 0.50 wt %	96.11 ± 0.03	$231.25 \pm 3.18^{\#}$	$71.17 \pm 1.22^{\#}$	$0.0185 \pm 0.0022^{\#}$	165.52 ± 27.82
60 : 40 (30) $AgNO_3$ 0.75 wt %	$98.06 \pm 0.26^{\#}$	210.53 ± 9.95	$65.08 \pm 2.51^{\#}$	$0.0136 \pm 0.0033^{\#}$	$113.53 \pm 10.19^{\#}$
60 : 40 (30) $AgNO_3$ 1.00 wt %	$96.78 \pm 0.14^{\#}$	166.46 ± 6.39	$64.66 \pm 3.85^{\#}$	$0.0123 \pm 0.0061^{\#}$	$99.93 \pm 10.29^{\#}$

The values, which share the same letter in the same column, have a statistically significant difference ($P < 0.05$). The statistically significant difference ($P < 0.05$) between gelatin/PVA and neat gelatin hydrogels in the same irradiation dose is denoted with an asterisk (*). The statistically significant difference ($P < 0.05$) between the 60 : 40 hydrogels (30 kGy) with and without $AgNO_3$ is denoted with a hash (#).

abundant amount of hydroxyl groups at the side chain.[58] In Table I, the lowest value of swelling behavior is >100% and the highest is >200%. The swelling decreased as the amount of PVA increased and as the irradiation dose increased. The higher irradiation dosage applied to the gelatin solutions caused greater crosslink density of the gelatin hydrogels.[59] The hydrogel with AgNPs swelled better than the hydrogel without AgNPs because some radiation was spent to reduce Ag^+ to AgNPs, therefore the hydrogel without AgNPs was exposed to a greater irradiation dose, which created a reduced crosslink density of the hydrogels.[29]

Water Vapor Transmission Rate. WVTR, also known as moisture vapor transmission rate, is an important characteristic of wound dressings. When clinicians select dressings for wound management, they have to decide which type of dressing can maintain moisture for each type of wound.[60] The WVTR is an effective indicator of the moisture-retention capacity of several wound dressings.[37,38] Therefore, WVTR characterization is important for wound dressing testing.

Commercial dressings demonstrate WVTR values between 76 and 9360 $g/m^2/day$.[61] Wound dressing materials that have a proper WVTR can maintain the wound area at an appropriate moisture level. Queen et al.[62] demonstrated that a suitable WVTR of 2000–2500 $g/m^2/day$ could keep the wound area moistened to prevent over dehydration or exudation.

As shown in Figure 1, all of the hydrogels have high WVTR. The 60 : 40 hydrogels, which were irradiated at 50 kGy, have the lowest WVTR (~3000 $g/m^2/day$). A neat gelatin hydrogel, irradiated at 30 kGy, has the highest WVTR (~5400 $g/m^2/day$). When the irradiation dose or amount of PVA increased, the WVTR decreased. Although the WVTR of the hydrogels is higher than the recommendation, the dressing with a high WVTR can prevent the collection of exudates in the wound area.[63] In clinical situations, hydrogels are usually used as a primary dressing, attached to the wound area, then covered by a secondary dressing. Queen et al.[64] have demonstrated that the high WVTR of the hydrogels can be decreased by putting an adhesive bandage over the hydrogels.

Tensile Test. Hydrogels, which are the soft and porous structure that filled with a lot of fluid, have viscoelastic properties.[65] Because of their characteristics, the mechanical testings of hydrogel are seriously hard.[66] The standard tests for plastics and rubber were adapted for determine the mechanical properties of the hydrogels in this research.

Wound dressings are products that must be attached to the skin, so it should be able to follow the contours of the skin and not tear easily when stretched. Therefore, the mechanical properties of hydrogels were tested by tensile testing. The stress and percentage strain at maximum load of as-prepared hydrogels were determined using a universal testing machine (Lloyd). Table I gives the results of stress at maximum load of the hydrogels. The stress at maximum load of the hydrogels with AgNPs decreased when the amount of $AgNO_3$ increased. In Table I, the percentage strain at maximum load of the hydrogels increased when the amount of PVA increased, but decreased when the irradiation dose increased. This might predict that the toughness or viscoelastic properties of PVA, which is thermoplastic, can improve the brittleness of gelatin.[67] A higher irradiation dose caused a higher degree of crosslink of the neat

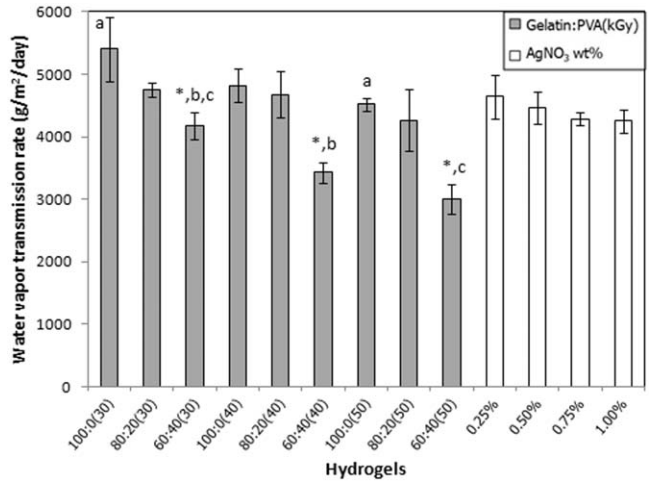

Figure 1. WVTR of hydrogels. The bars, which share the same letter, indicate that there is a statistically significant difference ($P < 0.05$). The statistically significant difference ($P < 0.05$) in WVTR between the gelatin/PVA and neat gelatin hydrogel in the same irradiation dose is denoted with an asterisk (*).

gelatin hydrogels, thus resulting in a decrease in the ductile of the hydrogels. The percentage strain at maximum load of the hydrogels with AgNPs decreased when the amount of $AgNO_3$ increased. Ag^+, which bound with —OH, C═O, and —NH groups of gelatin[68], and —OH group of PVA, may has created a scattered interruption of nanoparticles on the gelatin/PVA matrix, thus leading to less strain of the hydrogels with AgNPs.[69]

This result was consistent with our preliminary study.[70] The stiffness of the 80 : 20 hydrogel was less than the stiffness of the gelatin hydrogel, when irradiated at 40 kGy. The addition of PVA made the hydrogels less rigid, more flexible, so they could be pulled longer than the neat gelatin hydrogels. Moreover, the tensile test results have a wide SD, which might be caused by the inexact received dose of hydrogels. The means ± SD of the received irradiation doses, which were targeted at 30, 40, and 50 kGy, were 30.0 ± 2.5, 41.5 ± 2.7, and 50.8 ± 0.7 kGy, respectively. The stress at 50% strain[71,72] were also examined. The stress of the hydrogels with AgNPs was less than the stress of the hydrogels without AgNPs, as shown in Supporting Information Figure S2. The interruption of AgNPs in the structure of hydrogels could be responsible for this occurrence.

Stress-strain curves of hydrogels are shown in Supporting Information Figure S3. The beginning of curve demonstrated almost straight slope and then sharp drop at yield point. The sharp drop may be the results from defects that happened from the expanding cracks in material.[73] The curves revealed the ductile behavior of hydrogels. However, the stress-strain curves of some hydrogels had several sharp drops. The incomplete cracks that repeatedly happened could be the cause of these drops.[74]

***In Vitro* Biodegradation.** Lysozyme is an enzyme that is found in human wounds and blister fluid.[75] The hydrogels were incubated in PBS with lysozyme to mimic the wound environment, which can degrade the dressing. Results of remaining weight percentage after *in vitro* biodegradation of the hydrogels without AgNPs for 24 h are shown in Figure 2(a). A higher irradiation dose caused a greater remaining weight of the hydrogels. The hydrogels containing PVA had more durability, the same as the results from our preliminary study that showed that the weight loss of neat gelatin hydrogels was more than that of the gelatin/PVA hydrogels.[70]

The 60 : 40 hydrogels, which were irradiated at 30 kGy, were selected to create the AgNP/gelatin/PVA hydrogels. This proportion was selected because of its optimal water holding capacity, highest percentage strain at maximum load, and high remaining weight after *in vitro* biodegradation. Their properties were suitable to use as the hydrogel sheet wound dressing. The AgNP/gelatin/PVA hydrogels were then tested for the *in vitro* biodegradation by dividing the time intervals into 6, 12, 18, and 24 h. World Health Organization (WHO) has suggested that the daily treatment of burn wounds should change the wound

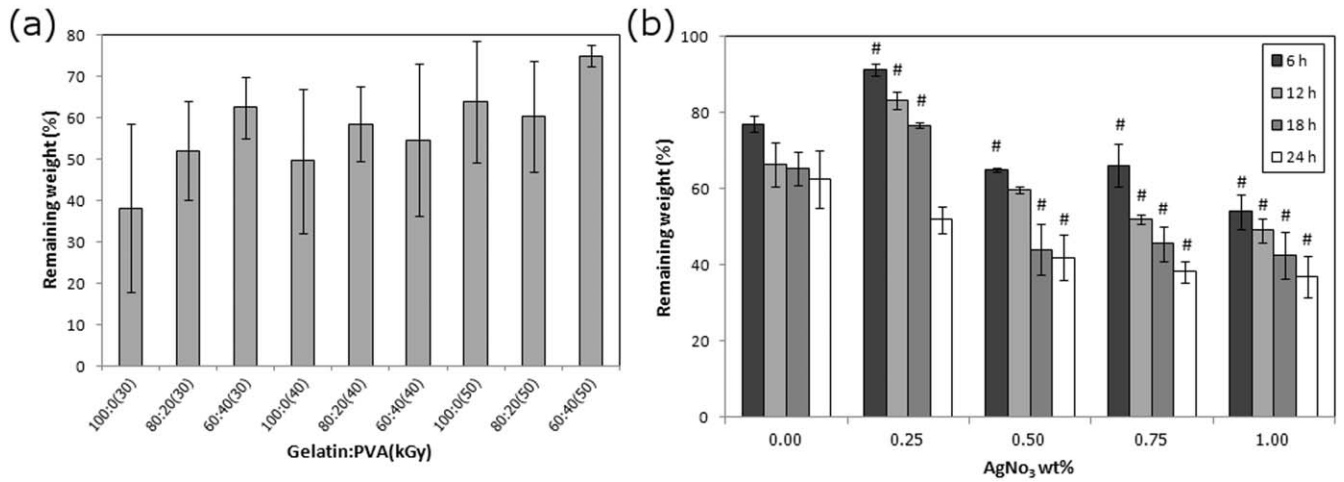

Figure 2. The remaining weight percentage of (a) hydrogels without AgNPs after *in vitro* biodegradation for 24 h and (b) of AgNP/gelatin/PVA hydrogels compared with gelatin/PVA hydrogels without AgNPs. The statistically significant difference ($P < 0.05$) in remaining weight percentage between the hydrogel with and without $AgNO_3$ at the same incubation time is denoted with a hash (#).

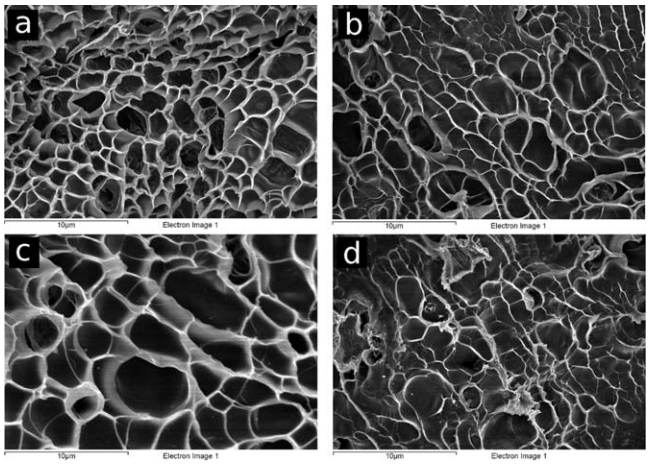

Figure 3. Representative SEM images (scale bar = 10 μm) of AgNP/gelatin/PVA hydrogels with (a) $AgNO_3$ 0.25 wt %, (b) $AgNO_3$ 0.50 wt %, (c) $AgNO_3$ 0.75 wt %, and (d) $AgNO_3$ 1.00 wt %.

dressing daily or twice daily.[76] This suggestion means the duration of covering of the wound dressing is 12 h or 24 h. In Figure 2(b), after 24 h of incubation with lysozyme in PBS, the hydrogels with AgNPs had a remaining weight of about 37–52%. However, at 12 h, the remaining weight was higher (around 49–83%), which is sufficient for covering the wound area because the hydrogel was used as the primary dressing with a secondary dressing covering it.

Gel Fraction. The gel fraction of hydrogels was investigated to indirectly estimate the degree of crosslinking. The gel fractions were ~between 65 and 89%, which is shown in Table I. The gel fraction of the hydrogels without AgNPs increased with increasing irradiation dose. A higher gel fraction was caused by a higher irradiation dose, thus resulting in an increase in the degree of crosslink. The hydrogels with higher PVA content had more gel fraction. These results can be related to the differences in the dissolution properties of gelatin and PVA.[77] The gel fraction of AgNP/gelatin/PVA hydrogels showed that hydrogels with more $AgNO_3$ had less gel fraction. These outcomes corresponded to the binding of Ag^+ with the functional groups of gelatin and PVA, which was described in the previous section.

SEM Images. Wound dressings, which are porous, are permeable for water vapor and wound exudates. The permeability of wound exudate can prevent bullae formation.[78] In this study, as seen in Figure 3, all hydrogels had porous network structures. The approximate pore diameter was between 1 and 5 μm. The AgNPs in the structures of hydrogels were not observable in the SEM images because microscales in the SEM images are larger than the nanosized silver particles.

FTIR Spectra. Hydrogels at 100 : 0, 60 : 40, and 60 : 40 with $AgNO_3$ 1.00 wt % were selected for analysis by FTIR spectroscopy. All hydrogels were irradiated at 30 kGy. As shown in Figure 4, FTIR spectrum of neat gelatin presented a peak at 3289 cm^{-1} corresponding to —NH stretching of the secondary amide, C═O stretching at 1630 cm^{-1}, and N—H bending at 1528 cm^{-1}. The FTIR spectrum of the gelatin/PVA hydrogel was similar to the spectrum of neat gelatin because the amount of gelatin (60) was much more than PVA (40). However, the peaks at 3289 cm^{-1}, in the spectra of neat gelatin and gelatin/PVA hydrogel, were broad indicating hydrogen bonds between the hydroxyl groups of PVA, gelatin and amide groups of gelatin. FTIR spectrum of the AgNP/gelatin/PVA hydrogel exhibited a broad peak compared to other spectrum. This might be due to the effect of reduced AgNPs on the functional groups of the polymers.[35]

Characterization of AgNP Formation

UV–vis Analysis. The optical property of AgNPs is generally used to confirm the formation of AgNPs with the peak of the characteristic SPR band of AgNPs being around 410–430 nm.[36,79–82] There was bare absorbance at 410–430 nm for the hydrogels without $AgNO_3$, and there were peaks of AgNPs from the hydrogels with $AgNO_3$ (0.25–1.00 wt %). The hydrogels with a greater percentage of loaded $AgNO_3$ had a greater intensity characteristic peak. This outcome could be due to the AgNPs producing higher yields.[79] UV–vis spectra are shown in Supporting Information Figure S4.

TEM and EDX. Images of the AgNPs, which are shown in the top row of Figure 5, were investigated by using TEM. The sizes of the AgNPs are quite small. The size distribution of the AgNPs is shown in the bottom row of Figure 5. The largest particles have a diameter of 8 nm, which is good for antibacterial performance. The nanoparticles with diameters <10 nm have high efficiency for antibacterial activity. These small particles can attach to bacteria directly.[31] The TEM images in Figure 5 indicate that gelatin and PVA, which make up the structure of the hydrogel, can stabilize the silver in the form of nanoparticles. The polymer can prevent the aggregation of AgNPs. EDX analysis, which confirms the presence of the silver element in the hydrogel structure, was also investigated. The EDX spectrum displayed characteristic L-series peaks of silver, which were located between 2.63 keV to 3.82 keV.[83] EDX peaks of silver (Ag) were observed in every hydrogel samples. The results are shown in Supporting Information Figure S5. The TEM images indicated that the AgNPs in this study were very small. The functional groups of the hydrogel polymers bind to the AgNPs, therefore the nanoparticles do not agglomerate into a large size. This is one of the advantages of the *in situ* synthesis of AgNPs. Most of the preparation methods for the hydrogel with

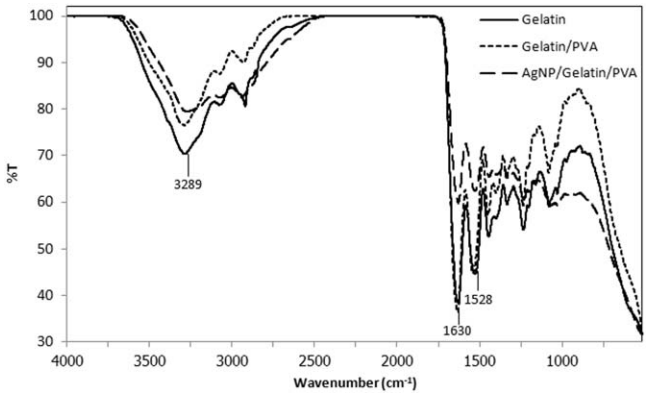

Figure 4. FTIR spectra of gelatin, gelatin/PVA, and AgNP/Gelatin/PVA hydrogels.

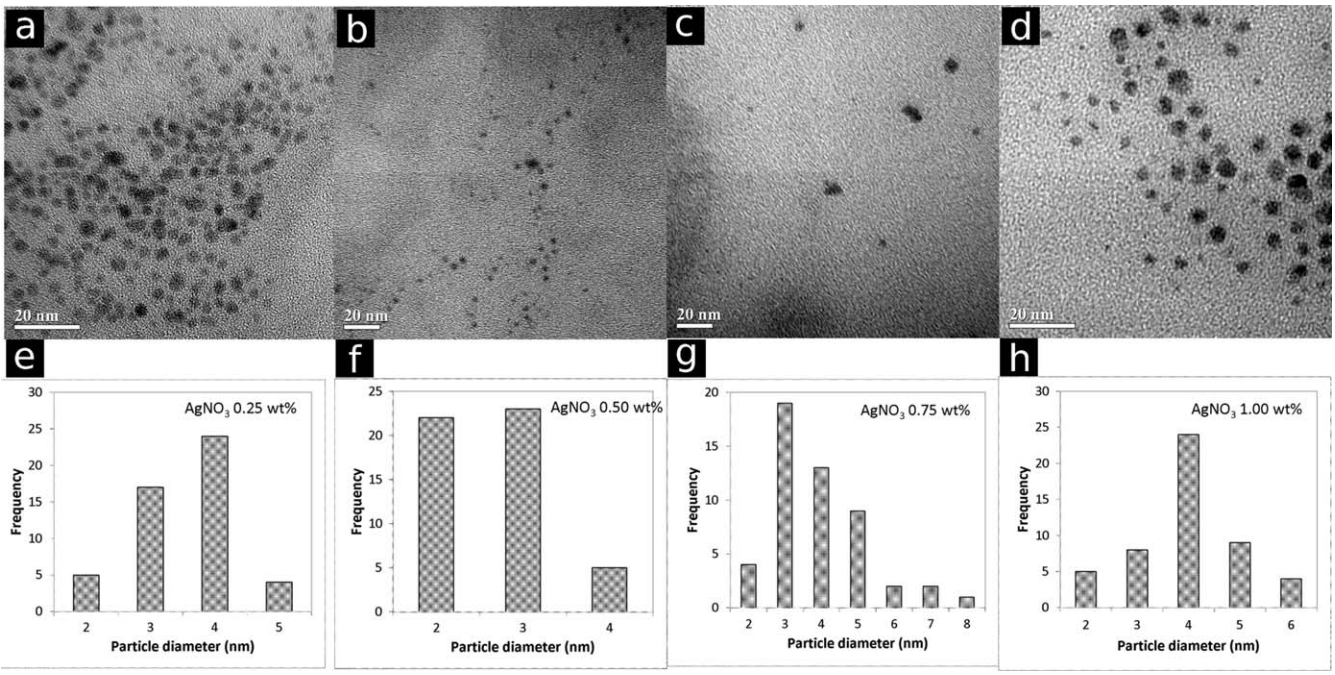

Figure 5. Top row: Selected TEM images (scale bar = 20 nm) of AgNPs in AgNP/gelatin/PVA hydrogels and bottom row: size distribution of AgNPs in AgNP/gelatin/PVA hydrogels: (a) and (e) $AgNO_3$ 0.25 wt %, (b) and (f) $AgNO_3$ 0.50 wt %, (c) and (g) $AgNO_3$ 0.75 wt %, and (d) and (h) $AgNO_3$ 1.00 wt %.

nanoparticles usually start with the prefabrication of the nanometal, and are followed by the incorporation of nanoparticles into the hydrogel ingredient.[84] Later, the agglomeration of AgNPs might occur, and explode the abundant of silver to the body which can cause toxicity.[85,86]

Silver-Release Assay. This study investigated the release characteristics of silver by mimicing the wound dressing. Hydrogels were placed on a CA sheet on the body of a Franz cell. This method made the hydrogels release silver from only one side of the material, same as the wound dressing released the drug from only one side that attach the wound area. In Figure 6, the release profile shows initial rapid release, followed by slow release after 6 h. The Ag^+ release in the earliest stage of the experiment originated from the silver particles at the surface of the hydrogels. The Ag^+ released quickly and did not require the diffusion process to migrate from the inside of the hydrogels. Next, the majority of released Ag^+ transported from the inside of the hydrogels to the surface and the remaining Ag^+ within the hydrogel structure needed diffusion to reach the surface of the hydrogels.[87]

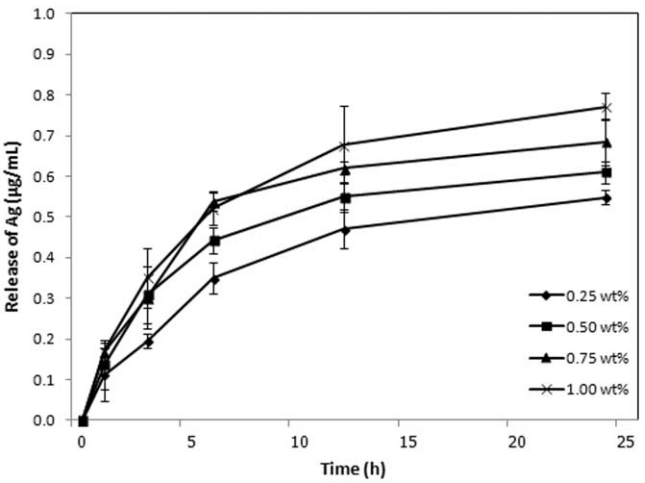

Figure 6. Release profiles of silver from AgNP/gelatin/PVA hydrogels.

Cytotoxicity and Antibacterial Testing

Indirect Cytotoxicity Testing. The ability to kill bacteria is an outstanding characteristic of wound dressings with antibacterial agents, but consideration for the cytotoxicity to human cells is also important. Therefore, we investigated the indirect cytotoxicity to test our hydrogels for the safety of the NHDF. In Figure 7(a), all hydrogels have cell viability greater than 80%, which indicated that they are safe for NHDF. Our preliminary study showed that the hydrogels were still safe with more gelatin and a higher irradiation dose.[70]

Antibacterial Activity. Counting colonies of bacteria is an effective way to determine the number of viable and productive bacteria. As seen in Figure 7(b), all hydrogels with AgNPs have almost 100% anti-bacterial efficacy, except *E. coli*, which has high efficacy also (>90%). Table II gives the data about the diameter of the hydrogel disc after the experiments and the clear zone. The gelatin/PVA hydrogels without $AgNO_3$ and the drug pellets of Gentamicin 10 μg and Vancomycin 30 μg were used as the negative control and the positive control, respectively. There were slightly clear zones around the AgNP/gelatin/PVA hydrogels, but there were no clear zone around the $AgNO_3$ 1.00 wt % hydrogels. Many AgNP/gelatin/PVA hydrogels were sloppy after the test because reduction of AgNPs reduced the

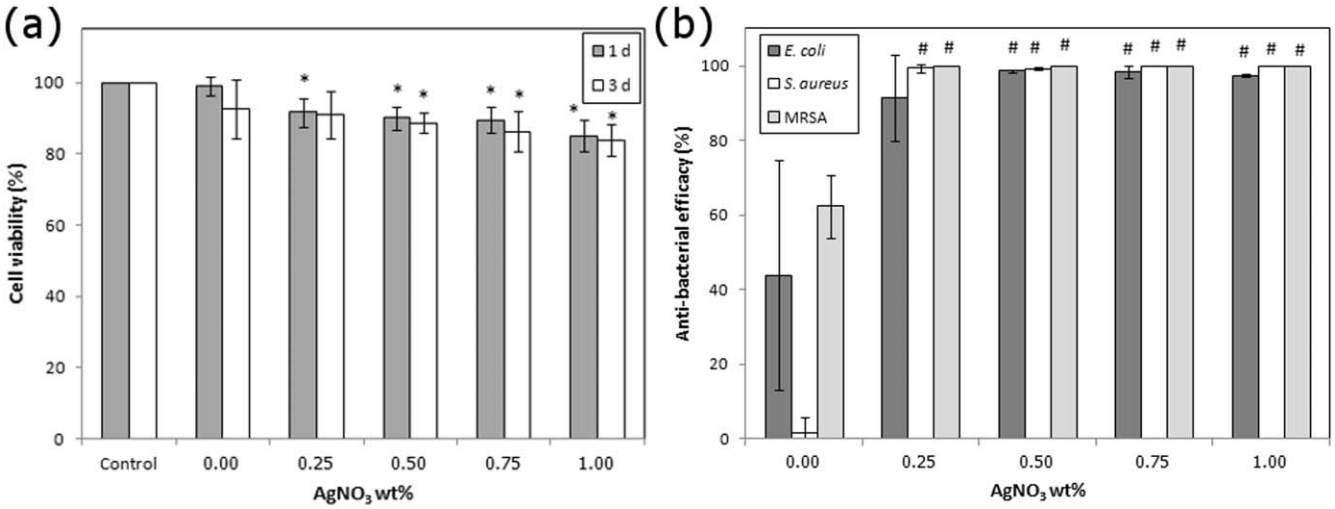

Figure 7. (a) Viability of NHDF that was cultured for 1 d (24 h) and 3 d (72 h) and (b) percentage of antibacterial efficacy of *E. coli*, *S. aureus*, and MRSA. The statistically significant difference ($P < 0.05$) in cell viability between the hydrogel and control group is denoted with an asterisk (*). The statistically significant difference ($P < 0.05$) in antibacterial efficacy between the hydrogel with and without $AgNO_3$ is denoted with a hash (#).

Table II. Average Diameter of the Hydrogel Disc and Clear Zone

Disc	Diameter of the disc before test (mm)	Diameter of the disc after test (mm)			Clear zone (mm)		
		E. coli	*S. aureus*	MRSA	*E. coli*	*S. aureus*	MRSA
Gelatin/PVA	15 ± 0	15 ± 0	15 ± 0	15 ± 0	0 ± 0	0 ± 0	0 ± 0
Gelatin/PVA $AgNO_3$ 0.25 wt %	15 ± 0	15 ± 0	15 ± 0	16 ± 0	1 ± 0	1 ± 0	1 ± 0
Gelatin/PVA $AgNO_3$ 0.50 wt %	15 ± 0	18 ± 0	18 ± 0	19 ± 0	0 ± 0	1 ± 0	0 ± 0
Gelatin/PVA $AgNO_3$ 0.75 wt %	15 ± 0	27 ± 2[a]	20 ± 0	24 ± 0[a]	0 ± 0	2 ± 0	0 ± 0
Gelatin/PVA $AgNO_3$ 1.00 wt %	15 ± 0	24 ± 3[a]	26 ± 2[a]	26 ± 1[a]	0 ± 0	0 ± 0	0 ± 0
Gentamicin 10 μg	7 ± 0	7 ± 0	7 ± 0	7 ± 0	5 ± 0	5 ± 1	1 ± 0
Vancomycin 30 μg	7 ± 0	-	-	7 ± 0	-	-	6 ± 1

[a] The discs were sloppy.

degree of crosslink of the hydrogels. However, there was no bacterial growth in area of the sloppy hydrogels. Thus the area of the sloppy hydrogels could be claimed as the clear zone. The NB agar under the gelatin/PVA hydrogels without $AgNO_3$ had an opaque appearance, indicated the presence of bacteria growth, therefore it could hardly be defined as a clear zone. Photographs of zone of inhibition test are shown in Supporting Information Figure S6.

The length of the clear zone hardly appeared while the percentage of antibacterial efficacy was very high. The conflict was due to the inhibition of bacterial growth on the agar, which was related to the diffusion of the released Ag^+ from the hydrogels. It is important to note that the hydrogels in the antimicrobial activity assays were shaken; therefore part of Ag^+ was released by the shaking force.

CONCLUSIONS

The AgNP/gelatin/PVA hydrogels were successfully synthesized by the gamma irradiation method. The hydrogels had appropriate properties for use as wound dressings: water holding capacity, WVTR, and remaining weight after *in vitro* biodegradation. The results of the physical properties testing indicated that the addition of PVA can improve mechanical properties and durability of the hydrogels. The degree of crosslink of the hydrogels was found to increase with an increase in the irradiation dose. Meanwhile, the degree of crosslink was found to decrease with an increase in the amount of $AgNO_3$. The AgNPs were successfully *in situ* synthesized by induction of $AgNO_3$. The existence of AgNPs in the hydrogels was confirmed by TEM, UV–vis, and EDX. The AgNP/gelatin/PVA hydrogels were tested for antibacterial activity against *E. coli*, *S. aureus*, and MRSA and indirect cytotoxicity to fibroblasts. The results showed that the hydrogels can be used against bacteria effectively, and are safe for human cells. The hydrogel with the highest $AgNO_3$ content (1.00 wt %) had the greatest antibacterial property, but it had the worst mechanical properties. The hydrogel with the lowest $AgNO_3$ content (0.25 wt %) had better mechanical properties and greater remaining weight after 24 h of degradation. The gelatin/PVA hydrogel with low $AgNO_3$

(0.25 wt %) content has potential to be developed as a disposable sterile antibacterial wound dressing.

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