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Hydrogels Containing Silver Nanoparticles for Burn Wounds Show Antimicrobial Activity Without Cytotoxicity

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ABSTRACT: This research introduces a novel dressing for burn wounds, containing silver nanoparticles in hydrogels for infected burn care. The 2-acrylamido-2-methylpropane sulfonic acid sodium salt hydrogels containing silver nanoparticles have been prepared via ultraviolet radiation. The formation of silver nanoparticles was monitored by surface plasmon bands and transmission electron microscopy. The concentration of silver nitrate loaded in the solutions slightly affected the physical properties and mechanical properties of the neat hydrogel. An indirect cytotoxicity study found that none of the hydrogels were toxic to tested cell lines. The measurement of cumulative release of silver indicated that 70%–82% of silver was released within 72 hr. The antibacterial activities of the hydrogels against common burn pathogens were studied and the results showed that 5 m*M* silver hydrogel had the greatest inhibitory activity. The results support its use as a potential burn wound dressing. © 2013 Wiley Periodicals, Inc. J. Appl. Polym. Sci. **2014**, *131*, 40215.

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INTRODUCTION

Patients with serious burn wounds require specialized treatment to minimize trauma and mortality. Sepsis and wound infection are the major causes of morbidity and mortality.[1,2] Invasive wound infection is influenced by the type and quantity of microorganisms that colonize the burn wound. Well-known pathogens that infect the wound and are resistant to broad antimicrobial agents are gram-positive methicillin-resistant *Staphylococcus aureus* (MRSA) and gram-negative *Pseudomonas aeruginosa*.[3] As more resistant pathogens have continued to develop, antibiotics have become less effective.

Silver has been found to be a useful therapeutic agent for preventing wound infection. Silver nitrate ($AgNO_3$), silver sulfadiazine, and nanocrystalline silver have the ability to reduce viability of antibiotic-resistant bacteria with varying efficacy. Nanocrystalline silver shows the most effective inhibitory action with a rapid inhibition rate, and it was efficient against a wider range of microbes, while $AgNO_3$ shows the least efficiency.[4] Silver sulfadiazine is more effective than $AgNO_3$ because it supplies silver ions over a longer period of time. Silver ion is less effective than nanocrystalline silver because it forms salts with halides resulting in an inactive silver form.[5] For example, silver ions form insoluble salts with chloride and the salt AgCl has a Ksp (25°C) of 1.77×10^{-10}.[6] AgCl will be precipitated and the active silver ions will be eliminated from the solution.

The inhibitory action of silver on bacterial cells is related to the strong interaction of silver with thiol groups present in key respiratory enzymes in bacteria.[7] In addition, silver interacts with structural proteins and binds with DNA, leading to inhibition of replication.[8] Microbial resistance to silver is rare,[5] supporting silver-based dressings as interesting candidates to use against infection for burn wounds.

Silver-based technologies provide benefits that facilitate wound healing. Studies report that nanocrystalline silver may play a role in facilitating the early phases of wound healing and enhancing cellular apoptosis.[9] Antimicrobial silver-based dressings may also cause cellular toxicity,[10] however, it has been found that low concentrations of silver nanoparticle have minimal acute mammalian cell toxicity.[11] It has previously been reported that silver nanoparticles were free of *in vitro* cytotoxicity and showed high antibacterial activities against multiresistant

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bacteria.[12] A slower silver ion release rate may also reduce the inhibition of wound-healing mechanisms.[10]

In caring for patients with burns, it is important to use suitable burn wound dressings. Various biologic, biosynthetic, and synthetic wound dressings are available for use. The selection of dressing depends on the wound bed, inherent properties of the dressings, and the therapeutic goals. Dressings play an important role as ideally they accelerate wound healing and create better healing conditions without any harm to the wounds. In 1979, Turner introduced the characteristics of an "ideal wound dressing" including: (1) the ability to absorb exudates and toxic components from the wound surface; (2) maintain high humidity at the wound/dressing interface; (3) allow gaseous exchange; (4) provide thermal insulation; (5) protect the wound from bacterial penetration; (6) be non-toxic; and (7) be removed easily without trauma to the wound.[13]

Hydrogels are popular candidates for use as dressings because of their highly absorptive properties at the wound site and ability to maintain a moist environment to promote wound healing as well as regeneration of defective or lost tissue.[14] Among the synthetic polymeric hydrogels, 2-acrylamido-2-methylpropane sulfonic acid (AMPS) hydrogel provides many interesting properties such as: hydrophilicity, high swelling capacity, lack of toxicity and biocompatibility.[15] Additionally, AMPS hydrogel has attracted interest as a wound dressing as it can be easily removed without causing harm and is transparent, allowing for visual monitoring of the wound.

Ultraviolet (UV) radiation is an effective technique that has been widely used to covalently crosslink polymer chains. It can produce stable three-dimensional hydrogel networks. The most common mechanism for UV curing is free radical polymerization. Its advantages include; a fast curing rate, low curing temperature, low energy consumption, low heat production, and easy process control. Moreover, cross-linking hydrogels via UV irradiation is applicable to all shape, size, and thickness of hydrogel, enabling modification to match any shape and size of the burn wound.

The preparation of AMPS hydrogel containing silver nanoparticles (silver hydrogel) for use as potential antibacterial wound dressings have been proposed in this study. The AMPS monomer was cross-linked with UV irradiation via free radical polymerization. The physical and mechanical characteristics of the hydrogels were studied to evaluate their practical usefulness as dressings. Antibacterial activity against gram-positive *S. aureus* and MRSA as well as gram-negative *P. aeruginosa* were studied in order to confirm the potential of the silver hydrogels for use with infected burns. Their cytotoxicity to L929 (mouse fibroblast) and normal human dermal fibroblast (NHDF) cells were also determined.

EXPERIMENTAL

Materials

AMPS was purchased from Merck Chemicals, Germany. *N*-*N*′-methylenebisacrylamide (MBA) and 1-hydroxycyclohexyl phenyl ketone were purchased from Sigma-Aldrich. $AgNO_3$ (99.998 % purity) was purchased from Fisher Scientific (USA). Anhydrous sodium hydroxide (NaOH) pellets and hydrochloric acid (HCl) (37% w/w) were of analytical grade and purchased from Carlo Erba (Italy). All other chemicals were of analytical reagent grade and used without further purification.

Synthesis of Silver Hydrogel

$AgNO_3$ was dissolved in 40% 2-acrylamido-2-methylpropane sulfonic acid sodium salt (AMPS-Na^+) aqueous solution (pH 7.0 ± 0.1) in the presence of a cross-linker, MBA (0.1% mol/mol monomer) and an initiator, hydroxycyclohexyl phenyl ketone (0.01% w/w of monomer) to obtain the desired concentration (1, 2.5, 5, 10, 20, 50, or 100 m*M*) followed by stirring at room temperature for 30 min to obtain homogeneous solutions. Five milliliters of each mixture were loaded into 5×10 cm^2 nylon bags and the contents were sealed. The bags were then exposed to radiation under UV lamps (wavelength range between 280 and 390 nm, maximum intensity of 18.16 mW cm^{-2}, at a distance of 10 cm from the lamp) for 10, 30, or 120 min to allow formation of the cross-linked hydrogel pad. Neat hydrogel was similarly produced without addition of $AgNO_3$.

Characterization of Silver Nanoparticle Formation of Hydrogels

The neat hydrogel and silver hydrogel pads were cut into 3×4 cm^2 pieces and the formation of silver nanoparticles was monitored by surface plasmon bands using UV–Vis spectrophotometry on a Shimadzu UV-2550 UV–Vis spectrophotometer.

The silver hydrogels were cut into discs (15 mm in diameter) and immersed in deionized (DI) water (40 mL) at 35°C with agitation (60 rpm) for 24 hr to obtain the silver release solutions. The morphology and size of silver nanoparticles in the solutions were identified by transmission electron microscopy (TEM) using a JEOL JEM-2100 transmission electron microscope.

Characterization of Physical Properties of Hydrogels

To measure the equilibrium degree of swelling (EDS), a hydrogel pad was cut into 1×1 cm^2 and was weighed (w_1) followed by immersion in 40 mL of simulated body fluid (SBF, pH 7.4) solution.[16] After 72 hr immersion at room temperature, the swelled hydrogel was wiped with tissue paper and weighed (w_2). The percentage of EDS was calculated as follows:

$$\text{EDS}\,(\%) = \frac{w_2 - w_1}{w_1} \times 100\%$$

To determine gel fraction, the hydrogels were cut into 0.5×0.5 cm^2 pieces and dried in an oven at 60°C for 24 hr to obtain constant weights (w_0). Each dried specimen was placed in a tea bag and immersed in DI water at 121°C for 4 hr in an autoclave. The hydrogels were then dried again at 60°C for 72 hr to obtain the constant weight after the extraction (w_E). The gel fraction was determined by the percent gelation using the equation below.

$$\text{Gelation}\,(\%) = \frac{w_E}{w_0} \times 100\%$$

To assess swelling ratio, the hydrogels were cut into 0.5×0.5 cm^2 pieces and dried in an oven at 60°C for 24 hr to obtain the

constant weight (w_d). Each dried specimen was immersed in 15 mL of SBF at 37°C with agitation (60 rpm). At specific time intervals, the samples were gently wiped with tissue paper and were weighed (w_s). The swelling ratio was determined by the following equation:

$$\text{Swelling ratio}\,(\%) = \frac{w_s - w_d}{w_d} \times 100\%$$

To examine moisture retention capacity, each hydrogel pad was cut into 0.5×0.5 cm^2 pieces, put individually in a plastic cup and the initial weight (w_i) at room temperature was measured. At different time intervals, the plastic cup was weighed (w_t). Moisture retention capability (% Rh) was identified as the water retained within the hydrogel and calculated as follows:

$$\text{Rh}\,(\%) = \left(\frac{w_t}{w_i}\right) \times 100\%$$

The measurement of water vapor transmission rate (WVTR) was employed according to the monograph of the European Phamacopoeia. The moisture permeability of the hydrogel was determined from the weight loss of cylindrical bottle (13 mm in diameter) containing 10 mL of DI water. The bottle was capped on the mount with a hydrogel disc (15 mm diameter), sealed with parafilm and kept in an oven at 35°C for 24 hr. The WVTR was calculated using the following formula:

$$\text{WVTR} = \frac{(w_i - w_t)}{A \times 24} \times 10^6 \ \text{g m}^{-2}\,\text{h}^{-1}$$

where, A is the area of the bottle mount (mm^2), w_i and w_t are the weight of bottle before being placed in the oven and after being removed from the oven, respectively.

Characterization of Mechanical Properties of Hydrogel

The mechanical properties of the neat hydrogel and silver nanoparticle infused hydrogels were evaluated in term of tensile strength and percentage of elongation at break. The hydrogels were cut into 5×1 cm (thickness of 0.12 cm) pieces, and the mechanical properties were measured using a 500 N load and cross-head speed of 50 mm min^{-1} on a Lloyd Universal testing machine model LRX.

Silver Release from Hydrogels

To measure the amount of silver released in physiological conditions, the silver hydrogels (with UV radiation exposure of 120 min) were cut into discs (15 mm in diameter, 1.24 ± 0.10 in thickness, 260.0 ± 2.7 mg) and each individual disc was immersed in a bottle containing 50 mL of SBF solution at 37°C with agitation at 60 rpm. At each time point the immersion solution was collected for investigation of cumulative release of silver. The 50 mL of SBF solution was replaced into the bottles before the next collection time point. The concentration of silver was measured using a Varian SpectrAA300. The total amount of silver content in hydrogels was measured using a same size hydrogel disc immersed in 50 mL of nitric acid solution (HNO_3).

Indirect Cytotoxicity Assessment

The indirect cytotoxicty assay was based on a protocol that was adapted from the ISO 10993-5 standard test method. The neat and silver hydrogels (1, 2.5, and 5 mM $AgNO_3$, with UV irradiation of 10 and 120 min) were cut into circular discs (5 mm diameter, 20 ± 0.7 mg), sterilized by immersing in 70% v/v ethanol. The extraction medium was prepared by immersing the individual hydrogel in 24-well plates that contained 2 mL of SFM for 1, 3, and 7 days. L929 (mouse fibroblast) cells were cultured in DMEM media (comprised of 10% fetal bovine serum [FBS], 1% L-glutamine, and 1% antibiotic and antimycotic [Invitrogen Corp., USA]). The cultures were incubated at 37°C and the media were changed every 2 days. The L929 cells were trypsinized and cultured with DMEM in 24-well tissue-culture polystyrene plates (TCPS; NunclonTM, Denmark) at a density of 5000 cells/well in DMEM overnight to allow cell attachment. The medium was removed and replaced with a serum-free media (SFM) overnight, then replaced by the extraction media of hydrogels, and the cells were again cultured overnight. The relative cell viability was determined using MTT assays. The cells were washed with PBS. A 300 μL of 0.5 mg mL^{-1} MTT was added to each well and the plate was incubated at 37°C for 10 min. This provided absorbencies within the linear range of the instrument (0.1–1.0) where the assay is most accurate.[17] The MTT solution was removed and 1 mL of dimethylsulfoxide (DMSO) solution (900 μL of DMSO : 125 μL of glycine buffer pH 10.5) was added. The plate was agitated for 10 min at 250 rpm and the absorbance of the solutions at 570 nm was measured by a Thermospectronic Genesis 10 UV–Visible spectrophotometer. The relative cell viability was calculated from the equation below:

$$\text{Relative cell viability}\,(\%) = \frac{\text{A570 of treated cells}}{\text{A570 of control cells}} \times 100\%$$

The cytotoxicity of NHDF cells was studied using a similar protocol as above. For NHDF, the cells were cultured in 24-well plates at a density of 10,000 cells/well. The treatment with MTT solution was carried out for 1 hr. The cytotoxicity of NHDF was studied only for the 3-day extraction solutions, otherwise the protocol was the same as the L929 study.

Antibacterial Activity Measurement

The antibacterial activity against *S. aureus* (ATCC 25923) was examined according to a modified shake flask method (ASTM E2149-01). The test samples were cut into discs (15 mm in diameter, 1.24 ± 0.10 cm in thickness, 260.0 ± 2.7 mg) and sterilized under UV lamps (FUNA-UV-LINKER FS800, Funakoshi, Japan) for 10 min. A colony of *S. aureus* was cultured in Tryptic Soy Broth (TSB, 2 mL) at 37°C for 2 hr with agitation (Orbital incubator shaker, GYROMAX^{TM} 737) at 250 rpm. The culture medium was transferred to 100 mL of TSB and incubated for 3–4 hr with agitation at 250 rpm until reaching an OD 600 of 0.6 ± 0.1. The cells were then collected and washed before being diluted with alkaline peptone water (APW) to 1 : 100 dilution (0.01×). Addition of 100 μL of this diluted cell culture solution into a flask containing 50 mL of APW produced 10^5 CFU mL^{-1} of cells. Each hydrogel was added into the culture flask, which was incubated at 37°C on a shaker (250 rpm). At the predetermined time points (0, 1, 3, 6, 12, and 24 hr), a 0.5 mL aliquot of each culture solution was diluted (10^{-1}, 10^{-2}, 10^{-3},...) and 100 μL of each diluted solution was spread on Tryptic

Table I. Photographs Illustrating Silver Hydrogels after Exposure to Different Times of UV Radiation. [Color table can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Concentration of $AgNO_3$ (mM)	UV irradiation period (min) 10	30	120
1.0			
2.5			
5.0			

Soy Agar (TSA) in duplicate and incubated at 37°C. After 24 hr, the number of colonies was counted manually and calculated taking into account the dilution factors. The number of colonies (CFU mL^{-1}) was expressed as the average of duplicate counts. The percent reduction was calculated using the following equation:

$$\text{Log reduction} = \text{Log}\,(\text{CFU/mL of control}) - \text{Log}\,(\text{CFU/mL of sample})$$

The antibacterial activity of 5 m*M* silver hydrogel against two major pathogens that infect burn wounds MRSA (wild type) and *P. aeruginosa* (ATCC 9027) was also assessed. The hydrogel was sterilized in an autoclave at 121°C for 15 min. The bacterial broth was prepared to a concentration of 10^6 CFU mL^{-1} using a similar protocol to the one described earlier for *S. aureus*. The startup concentration of cells for *P. aeruginosa* and MRSA was 10^6 CFU mL^{-1} compared with 10^5 CFU mL^{-1} for *S. aureus* so that the antibacterial activities of the hydrogel against resistant strains could be fully evaluated. The dressing disc sample was added into a flask containing APW and the antibacterial assay was carried out as described previously for *S. aureus*.

RESULTS AND DISCUSSION

Silver Nanoparticle Formation

When the original colorless AMPS-Na^+ solution was UV irradiated, it remained colorless as the neat hydrogel pad. In the case of the colorless $AgNO_3$ loaded AMPS-Na^+ solutions, after exposure to UV irradiation, the colorless hydrogel pads changed to a brown shade with the shade of brown color. The shade of brown was dependent on the concentration of $AgNO_3$ and also the time of exposure to UV radiation. Table I shows the appearance of the irradiated hydrogel pads. The higher the concentration of $AgNO_3$ and the longer the UV irradiation, the darker the color becomes. The neat hydrogel was colorless and transparent. The color change possibly indicates the formation of silver nanoparticles in the hydrogels.[18]

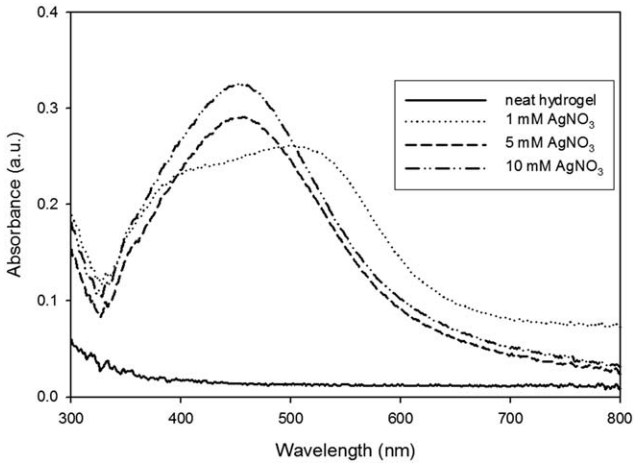

Figure 1. UV–Vis absorption spectra of the neat hydrogel and silver hydrogels (1, 5, and 10 m*M* silver hydrogel) with a radiation exposure time of 10 min.

The surface plasmon resonance absorption peaks around 430–450 nm can be used to identify the formation of silver nanoparticles. The decreased size of silver nanoparticles results in a shift in peak position to a shorter wavelength.[19] The neat hydrogel was used as a control and absorption peaks in the area of the silver nanoparticles was not observed, as expected. On the other hand, peaks in the area of 457.5 and 452.5 nm were observed for the 5 and 10 m*M* silver hydrogels, respectively (Figure 1), confirming that silver nanoparticles were formed. The peak position at 457.2 nm of the 5 m*M* silver hydrogel was shifted to 452.5 nm for the 10 m*M* silver hydrogel. The decrease in peak position indicates that the silver nanoparticle size of the 10 m*M* silver hydrogel was smaller than the silver nanoparticle size of the 5 m*M* silver hydrogel. For the 1 m*M* silver hydrogel, a peak at 500.5 nm was observed (Figure 1). This peak confirmed that the size of silver nanoparticles in 1 m*M* silver hydrogel was bigger than 5 and 10 m*M* silver hydrogels.

Figure 2 shows the spectra of the 5 m*M* silver hydrogels that were irradiated for different time intervals (10, 30, and 120 min).

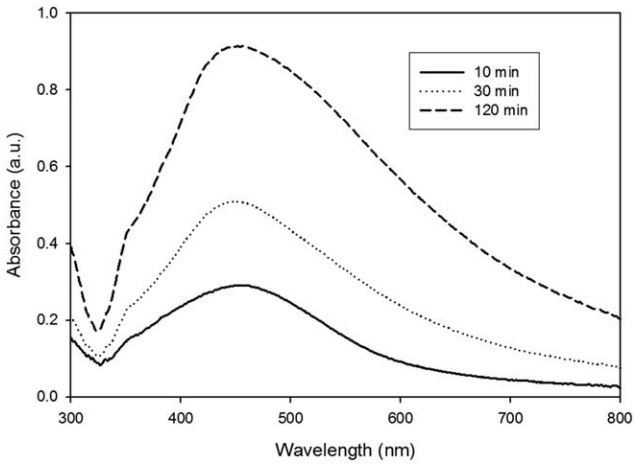

Figure 2. UV–Vis absorption spectra of 5 m*M* silver hydrogels that were irradiated for different time intervals (10, 30, and 120 min).

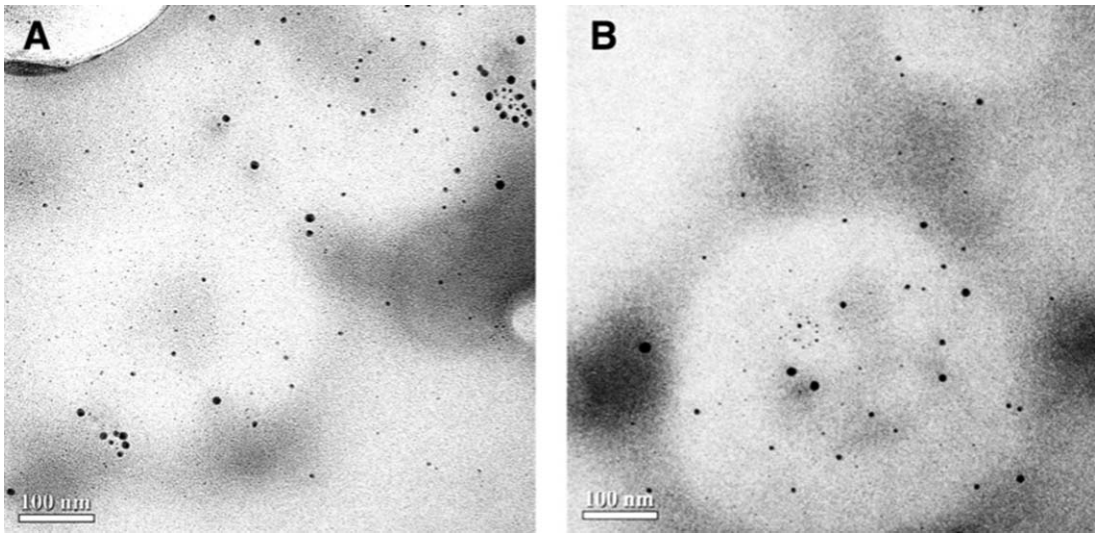

Figure 3. Selected TEM images (scale bar = 100 nm) from immersion solutions of silver hydrogels demonstrating the approximate size of the particles. (**A**: 2.5 m*M* silver hydrogel and **B**: 5 m*M* silver hydrogel, with UV radiation exposure of 120 min).

The size of silver nanoparticles depended on the radiation time intervals. The shift of peak from 457.5 nm of 10 min radiated hydrogel to 448.5 and 451 nm for 30 and 120 min irradiated hydrogels, respectively, indicated the smaller size of silver nanoparticles. The intensities of the spectra were found to be dependent on the concentration of $AgNO_3$ loaded into the hydrogels. This is similar to the color change of the irradiated hydrogel pads. The higher the concentration of $AgNO_3$ loaded in hydrogels the higher the intensity of UV–Vis spectra (Figure 1). Similarly, the longer radiation exposure time interval the higher the intensity of UV–Vis spectra, as shown in Figure 2. The higher intensity of the spectra indicates the presence of a larger amount of silver nanoparticles.

The TEM images of the silver release from 2.5, 5, and 10 m*M* silver hydrogels (irradiated for 120 min) indicated that the size of silver nanoparticles were in the range of 2–16 nm (Figure 3).

Physical Properties

The experiments were carried out with the neat hydrogel and silver hydrogels (1, 5, 20, 50, and 100 m*M*) with UV irradiation of 10 min. Table II shows the % EDS of the swelled hydrogels (in SBF solution) that range between 1663.8% and 1825.0%. For 50 and 100 m*M* silver hydrogels, the slight decrease in % EDS (1680.6% and 1663.8%, respectively) compared with the neat hydrogel and the lower concentration of silver hydrogels (1, 5, and 20 m*M*, 1805.8%–1915.1% EDS) can be observed. However, the slight difference between the % EDS of the neat hydrogel and various concentration silver hydrogels were not significant.

UV irradiation of AMPS-Na^+ solution resulted in the formation of an insoluble polymer network or gel. Table II shows % gelation of the neat hydrogel and various silver hydrogels (1, 5, 20, 50, and 100 m*M*, with UV irradiation of 10 min). The silver concentration in the hydrogels did not affect the % gelation as similar values of % gelation were observed in the range of 86.5%–88.6%.

Similar results (Table II) were found in the moisture retention experiment. At 6 hr there were no significant differences in moisture retention capacity between the hydrogels, with a range of 98.3%–98.8%. This again confirms that the neat hydrogel and the silver hydrogels were not different with respect to most physical properties.

Table II shows the WVTR of the neat and silver hydrogels (1, 5, 20, 50, and 100 m*M*, with UV radiation exposure of 10 min).

Table II. Physical and Mechanical Properties of the Silver Hydrogels

$AgNO_3$ content (mM)	EDS (%)	Gelation (%)	Moisture retention (%) at 6 h	WVTR (g m^{-2} h^{-1})	Tensile strength (MPa)	Elongation at break (%)
Neat	1813.6	86.5 ± 0.8	98.6 ± 0.1	94.4 ± 0.9	0.0628 ± 0.0078	439.7 ± 42.0
1.0	1825.0	87.3 ± 0.8	98.8 ± 0.1	100.7 ± 5.7	0.0652 ± 0.0109	525.6 ± 124.7
5.0	1915.1	88.0 ± 0.8	98.5 ± 0.2	99.0 ± 5.1	0.0527 ± 0.0036	441.4 ± 103.2
20.0	1805.8	88.6 ± 1.0	98.6 ± 0.1	94.0 ± 1.1	0.0573 ± 0.0103	544.2 ± 49.9
50.0	1680.6	88.0 ± 0.7	98.3 ± 0.4	103.6 ± 4.5	0.0516 ± 0.0043	433.1 ± 153.4
100.0	1663.8	86.6 ± 0.8	98.6 ± 0.2	107.0 ± 7.9	0.0525 ± 0.0052	530.3 ± 144.2

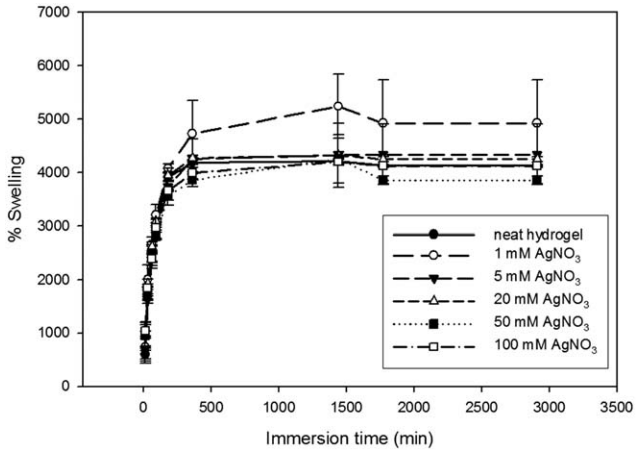

Figure 4. Swelling behaviors of the neat hydrogel and silver hydrogels immersed in SBF solution at 37°C.

The WVTR values range from 94.0 to 107.0 g m^{-2} h^{-1} and the concentration of silver did not affect the WVTR compared with the neat hydrogel.

Wound dressing materials ideally will be able to decrease the amount of body liquid loss by maintaining the humidity of the wound environment. Fortunately, the WVTR values of all hydrogels were lower than the evaporative water loss of second and third degree burn skin[20] which is 178 ± 5.5 g m^{-2} h^{-1} and 143.2 ± 4.5 g m^{-2} h^{-1}, respectively. This suggests that AMPS sodium salt hydrogels can be used to control body fluid loss and maintain the moist environment of burn wounds.

The swelling behavior in SBF (pH 7.4) of the neat hydrogel and silver hydrogels (1, 5, 20, 50, and 100 m*M*, with UV irradiation of 10 min) are shown in Figure 4. The experiment was carried out until the hydrogels reached their equilibrium. The different hydrogels did not show a significant difference in values for swelling behaviors, similar to their % gelation. These hydrogels would have a similar free volume inside their structure, which would relate to the swelling properties. Others have reported that % swelling can be used to determine the free volume inside the gel network.[21] The 10 min UV radiated hydrogel was selected to study the effects of silver content on properties of the hydrogels. However, the 120 min radiated hydrogel showed the best performance on surface plasmon resonance (Figure 2) and the physical properties of this dressing should be studied further in the future. The effects of UV radiation (10, 30, and 120 min) on hydrogel properties could also be investigated further.

Mechanical Properties

The mechanical properties of hydrogels as wound dressings are important to determine their actual applicability. Tensile strength and percentage of elongation were used to evaluate the mechanical properties of the silver AMPS salt hydrogels (1, 5, 20, 50, and 100 m*M*, with UV irradiation of 10 min). The silver loaded into the hydrogels did not cause a significant difference in the mechanical properties. The neat and silver nanoparticle infused hydrogels had tensile strength values in the range of 0.0516–0.0652 MPa and percentage of elongation in the range of 433.1%–544.2 % (Table II). All of the hydrogels are considered to have high tensile strength and percentage elongation values, which are advantageous properties for the handling of a wound dressing.

Cumulative Silver Release

From the surface plasmon band results, the formation of silver hydrogel with UV irradiation of 120 min showed the smallest size of silver nanoparticles. These silver nanoparticles are expected to provide better penetration of bacterial cells. As a result, silver hydrogels with UV radiation exposure of 120 min were chosen to test cumulative silver release. Table III shows the amount of silver theoretically available for release and the actual amount of silver (the free Ag^+ cation or the neutral silver nanoparticles) measured in the HNO_3. The cumulative release amount of silver hydrogels after immersion in SBF solution was also determined. For 1, 2.5, and 5.0 m*M* silver hydrogel, it is unclear why the actual amount of silver and the cumulative silver release values were larger than the amount of silver theoretically available. The results of 1 m*M* silver hydrogel were especially unexpected, and the theoretical silver amount was about half of the actual and cumulative silver amounts. There are two possible explanations for this. First, the silver was not homogeneously distributed within the sheet. Second, an error from measurement of very low silver concentrations in the solution occurred either from personal error or the instrument. In some cases applying the Beer–Lambert law directly in AAS is problematic because of atomization efficiency, sample matrix, non-uniformity of concentration, and path length of analyte atoms.[22] However, measurements for the silver released from hydrogels with a greater concentration of silver (5 and 10 m*M* silver hydrogel) were similar to the amount of silver theoretical available, and therefore were possibly accurate.

Table III. The Amount of Theoretically Available Silver, the Amount of Silver Released into HNO_3 (50 mL) Extracted from Silver Hydrogels (15 mm in Diameter, 1.24 ± 0.10 mm in Thickness, 260.0 ± 2.7 mg, with UV Radiation Exposure of 120 min), the Release Amount of Silver After Immersion of the Silver Hydrogels in SBF (50 mL) for 10 Days, and the Cumulative Silver Release

$AgNO_3$ (mM)	Amount of theoretical silver (ppm)	Amount of extracted silver (ppm)	Amount of silver released (ppm)	(%) cumulative silver release (ppm)
1.0	0.47	0.95	0.85 ± 0.01	89.5 ± 1.1
2.5	1.18	1.42	1.25 ± 0.13	88.0 ± 9.2
5.0	2.36	2.57	2.43 ± 0.01	94.5 ± 0.6
10.0	4.72	4.47	4.07 ± 0.08	91.1 ± 1.9

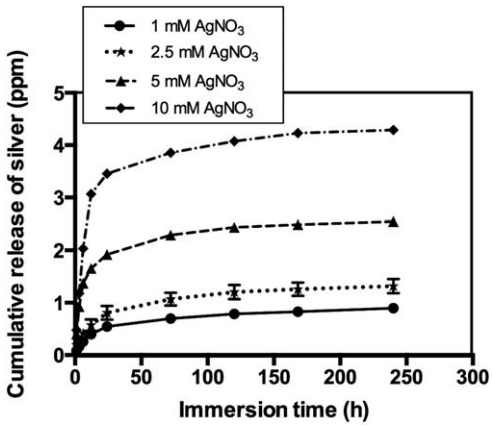

Figure 5. Cumulative release of silver from silver hydrogels immersed in SBF solution at 37°C.

It was found that the percentage of cumulative silver release of silver hydrogels ranged from 88.0% to 94.5% after 10 days of immersion. The release of silver from the hydrogels could be divided into two stages (Figure 5). There was an initial rapid release within the first 24 hr of immersion time, when 50.3% and 54.6% of silver was released from 1 and 2.5 m*M* silver hydrogels, respectively. For 5 and 10 m*M* silver hydrogels, the cumulative silver releases at 24 hr were 71.1% and 73.5%, respectively. The higher percentage of silver released from 5 to 10 m*M* silver hydrogel compared with 1 and 2.5 m*M* silver hydrogel could be explained by increased interaction between silver particles at higher concentrations. When silver nanoparticles or silver ions are trapped in the hydrogel sheet, they repulse other neighboring silver particles. So the high silver content hydrogels tend to release silver more rapidly into the solution compared with the lower silver content hydrogels. A terminal continuous slight silver release from 72 to 240 hr was observed for all of the hydrogels. It can be concluded that the hydrogels could be used as silver releasing wound dressing for up to 72 hr.

Indirect Cytotoxicity Evaluation

The investigation of indirect cytotoxicity of silver hydrogels was assessed to determine the effect of the dressings on cells in the wound area. L929 (mouse fibroblast) cells and NHDF cells were treated with the extraction media from the neat hydrogel and silver nanoparticle infused hydrogels. Figure 6 shows the relative viability of L929 that have been cultured with the dressing extraction media (1, 2.5, and 5 m*M* dressings with UV irradiation of 10 and 120 min) for 1, 3, and 7 days. None of the hydrogels were toxic to L929 cells as none of the relative viabilities were <90%. NHDF cells were cultured with the extraction media of the neat hydrogel and silver hydrogels (1, 2.5, 5, and 10 m*M*, irradiated for 120 min) for 3 days. The hydrogels were tested with NHDF cells for 3 days because they are recommend to be used as burn wound dressings for up to 72 hr according to the silver release results. None of the hydrogels were toxic to NHDF with none of the relative viabilities <95% (Figure 7). It can be concluded that the 1, 2.5, 5, and 10 m*M* silver hydrogels, which were irradiated for 120 min, were acceptable for use as non-toxic wound dressings at least up to 72 hr.

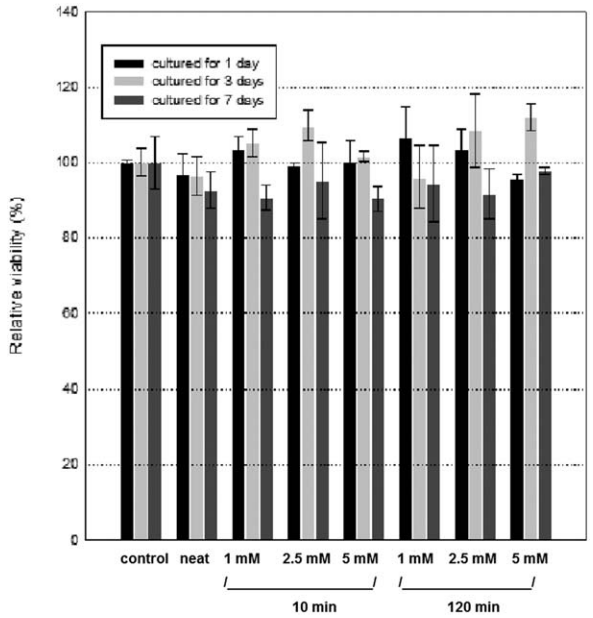

Figure 6. Indirect cytotoxicity evaluation of the neat hydrogel and silver hydrogels (with UV irradiation of 10 and 120 min). The viability of L929 cells that were cultured with extracted media obtained from the hydrogels were compared with those cultured in fresh culture medium.

When testing cytotoxicity, 5 mm diameter hydrogel discs were immerged in 2 mL of SFM. The silver theoretically available for release to the solution of 1, 2.5, 5, and 10 m*M* were 1.05, 2.63, 5.25, and 10.5 ppm, respectively. In general, silver ion concentrations >5 ppm will affect cells *in vitro*.[23] As a result, the silver released from 5 to 10 m*M* hydrogels would cause cytotoxicity to cells if the released silver specie were Ag^+. However, no cytotoxicity was found in both L929 and NHDF verifying that the silver released from the hydrogels was in forms other than silver ion or a mixture of forms including silver ion. We suggest one of the silver species released from silver hydrogels was silver nanoparticle, which generally has much less toxicity compared with silver ion.[24] In addition, it was confirmed by TEM images,

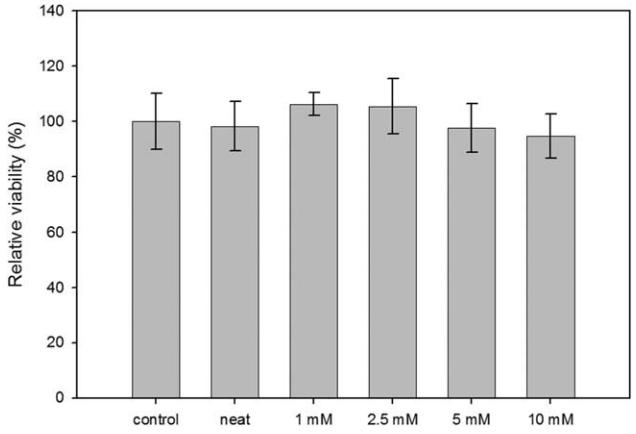

Figure 7. Indirect cytotoxicity evaluation of the neat hydrogel and silver hydrogels (with UV irradiation of 120 min). The viability of NHDF cells that were cultured in extracted media obtained from the hydrogels were compared with those cultured in fresh culture medium for 3 days.

Table IV. The Log Reduction of *S. aureus*, MRSA, and *P. aeruginosa* Treated with Silver Hydrogels

Bacteria	$AgNO_3$ (mM)	Log reduction at time periods (hr)				
		1	3	6	12	24
	Neat	−0.02 ± 0.03	0.10 ± 0.03	0.28 ± 0.04	0.34 ± 0.06	0.05 ± 0.01
S.aureus	1.0	−0.01 ± 0.38	0.05 ± 0.04	0.34 ± 0.11	0.32 ± 0.03	0.10 ± 0.03
	2.5	0.02 ± 0.04	1.13 ± 0.00	2.61 ± 0.10	1.10 ± 0.02	0.18 ± 0.12
	5.0	0.06 ± 0.02	1.86 ± 0.01	4.44 ± 0.76	>6.20 ± 0.00	>6.01 ± 0.00
MRSA	5.0[a]	0.00 ± −0.05	1.92 ± 0.01	3.45 ± 0.04	>6.41 ± 0.00	>6.28 ± 0.00
P. aeruginosa	5.0[a]	1.33 ± 0.09	>4.44 ± 0.00	>5.22 ± 0.00	>6.68 ± 0.00	>7.26 ± 0.00

[a] Denotes sterilization by an autoclaving at 121°C for 15 min.

as well as the surface plasmon resonance graphs, that the silver infused in the hydrogels are silver nanoparticles.

Antibacterial Evaluation

Antibacterial activity is one of the important properties for burn wound dressings. Infection may occur as a burn wound is healing or after it has been grafted. One of the most common causes of infection is gram-positive *S. aureus*.[25] It was used to select the best hydrogel dressing, which contains the lowest silver content yet retains good antibacterial activity. The log reduction of bacterial cell growth of *S. aureus* was used to investigate the antibacterial properties of silver hydrogels using the shake flask method. Table IV shows the log reduction of bacterial cell growth after 10^5 CFU mL^{-1} of *S. aureus* cells were treated with the neat hydrogel and silver hydrogels (1, 2.5, and 5 m*M* silver, with UV irradiation of 120 min) for different time periods. The low concentration silver hydrogels were selected to test antibacterial activity to determine the best hydrogel dressing. The best hydrogel is expected to have the lowest silver content (the lowest cytotoxicity to cells) as well as an effective antimicrobial activity. The neat hydrogel and 1 m*M* silver hydrogel showed similar results of antibacterial reduction. The log reduction of bacterial growth began at 3 hr and at 6 hr to 12 hr and the log reduction was between 0.28 and 0.34. After 24 hr of treatment, log reduction decreased to 0.05 and 0.10 for the neat hydrogel and 1 m*M* silver hydrogel, respectively, indicating that the neat hydrogel and 1 m*M* silver hydrogel decrease antibacterial activity for up to 24 hr. Neat hydrogel has no silver content, however, it showed slight antimicrobial activity up to 12 hr. This is likely due to the fact that neat hydrogel consists of AMPS-Na^+ salt. Sodium salt is well known to inhibit bacterial growth. It provides a hypertonic environment, which cause bacterial cells to lose water, shrink away from their cell walls, and cause cell death.

In the case of 2.5 m*M* silver hydrogel, after 3–12 hr a 1.10–2.61 log reduction of bacterial cell growth was shown. However, at 24 hr there was a decrease in antibacterial activity to 0.18 log reduction. For the 5 m*M* silver hydrogel, after treatment of 6–24 hr, the log reduction was 4.44–6.01. This indicates that 5 m*M* silver hydrogel is bacteriocidal at 6 hr against *S. aureus* because a log reduction >3 is considered bactericidal while log reduction <3 is generally considered bacteriostatic.[26] Figure 8 shows *S. aureus* viability (kill curve) after treatment with the hydrogels. It is clear that only the 5 m*M* silver hydrogel reduced the number of colonies to $<10^5$ CFU mL^{-1} after 3 hr of the treatment and the number of colonies continued to be reduced. Within 12 hr, the bacterial inoculum was reduced to below detection limit (10^2 CFU mL^{-1}). It can be concluded that 5 m*M* silver hydrogel had the most antibacterial activity within 24 hr of treatment. It was determined to be the best silver dressing for this study.

To serve as a ready to use sterile dressing for burns, the best hydrogel dressing, 5 m*M* silver hydrogel, was sterilized at 121°C for 15 min in an autoclave and was tested for antimicrobial activity to determine if heat will cause a change in antimicrobial activity. No sterilization technique was used for the antimicrobial testing of *S. aureus* initially, because it was a survey to check the antibacterial activity of the novel silver dressing and the sterilization process might change the antimicrobial activity.

The antibacterial activity assays against MRSA and *P. aeruginosa* were conducted to test the efficacy of the best hydrogel dressing (5 m*M* silver hydrogel) after sterilization by autoclave. The pathogen loads were increased to 10^6 CFU mL^{-1}, which is

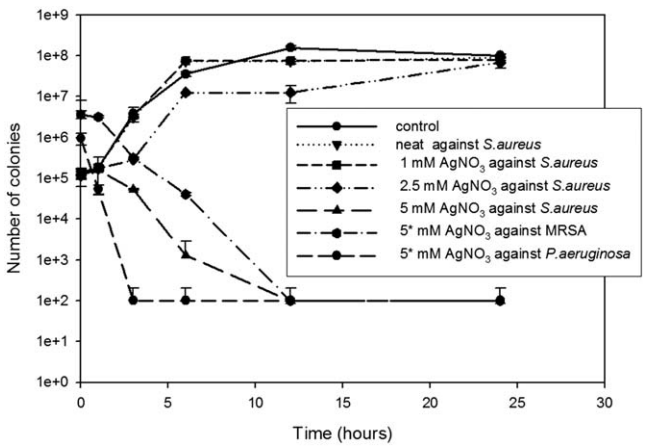

Figure 8. *Staphylococcus aureus* viability curves induced by the neat hydrogel and silver (1, 2.5, and 5 m*M*) hydrogels, and MRSA and *P. aeruginosa* viability curves induced by sterile 5 m*M* silver hydrogels. *Denotes sterilization by autoclaving at 121°C for 15 min.

higher than the infection point of 10^5 CFU g^{-1} of tissue, in order to determine inhibition by the hydrogel. The 1 and 2.5 m*M* silver loaded hydrogels were excluded from testing against these two pathogens because they showed poor antibacterial properties for *S. aureus*. For MRSA, the log reduction of 5 m*M* silver hydrogel (Table IV) was similar to that found for *S. aureus* indicating that 5 m*M* silver hydrogel provided the same antibacterial activity against gram-positive MRSA and *S. aureus*. In the case of gram-negative *P. aeruginosa*, the results show a faster inhibition rate as the log reduction was 4.44 after 3 hr treatment, therefore, 5 m*M* silver hydrogel is bactericidal at 3 hr. These results show that being autoclaved does not change the hydrogel dressing's antibacterial activity. Consequently, the autoclave should be the preferred method of sterilization because it is cost efficient and does not decrease antibacterial activity.

Figure 8 shows MRSA and *P. aeruginosa* viability after treatment with sterile 5 m*M* silver hydrogel. It was found that 5 m*M* silver hydrogel reduced the MRSA inoculum to $<10^5$ CFU mL^{-1} after 6 hr of treatment and the bacterial inoculum was continuously reduced after further treatment. Within 12 hr it was decreased to below the detection limit. This is the same inhibition rate of that observed for *S. aureus* treatment. In the case of *P. aeruginosa* the bacterial inoculum was $<10^5$ CFU mL^{-1} within 1 hr of treatment and after 3 hr, it was reduced to below the detection limit. The 5 m*M* silver hydrogel has potential antibacterial activity over gram-positive MRSA and *S. aureus* (within 12 hr) as well as gram-negative *P. aeruginosa* (within 3 hr). These properties support the 5 m*M* silver hydrogel for use as a novel antibacterial wound dressing.

If we infer that the tested hydrogel discs (1.5 cm in diameter) release silver in the solution of APW at the same rate as the release in SBF, at 3 and 12 hr, 5 m*M* silver hydrogel would release 0.93 and 1.65 ppm of silver, respectively. These silver amounts are extremely low but they showed potential antibacterial action. This can be explained by the size of the silver nanoparticles infused in the hydrogel, which were between 2 and 16 nm. Smaller sized silver particles provide a larger cumulative surface area to contact with bacterial cells. Also the silver nanoparticles remain active in the solution because they do not react with anionic ions to form inactive silver salts. A previous study showed the potential antibiotic properties of silver nanoparticles at very low concentrations. A 7–20 nm silver nanoparticle was tested against standard reference cultures and multidrug-resistant organisms and the minimum inhibitory concentration (MIC) of the silver nanoparticles was 0.78–6.25 ppm. When these silver nanoparticles were tested on human cells, cytotoxicity was not observed until concentrations >250 ppm.[27]

In our study, the novel silver hydrogel took 12 hr to inhibit the growth of the two gram-positive bacteria (MRSA and *S. aureus)*, and 3 hr to inhibit the growth of the gram-negative bacteria (*P. aeruginosa*). The inhibition rate is comparatively slow compared with the inhibition rate of a widely used silver coated dressing (Acticoat™), which inhibited the growth of variety of resistant strains within 30 min.[4] The greater silver content loaded in Acticoat™ (0.84–1.34 mg cm^{-2}) is about 20-fold higher than the concentration loaded into the novel silver hydrogel (0.054 mg cm^{-2}).[28] The high concentration of silver together with the nanoparticle size may provide a faster inhibition rate. However, researchers found that the silver coated dressing caused cell cytotoxicity for *in vitro* studies.[29–31] The high silver content in the dressing enhances the antimicrobial inhibition rate but it also increases cytotoxicity to cells. This novel silver hydrogel has low silver content and it has no significant cytotoxicity to cells. This is an advantage over the silver coated dressing. The balance between cytotoxicity and antimicrobial activity should always be considered to optimize the best conditions for wound healing.

CONCLUSIONS

Novel antibacterial wound dressings comprising of AMPS-Na^+ hydrogels containing silver nanoparticles have been successfully prepared via UV irradiation. The formation of silver nanoparticles was observed by the change in color from colorless (neat hydrogel) to brown shades for silver hydrogels after irradiation. The higher the concentration of silver in AMPS solution and the longer the UV irradiation time, the darker brown hydrogel sheets become. The monitoring of surface plasmon bands using UV–Vis spectrophotometry showed peaks around 450 nm that confirmed the nano-size of silver nanoparticles. The peak position was at 457.2 nm for the 5 m*M* silver hydrogel and 452.5 nm for the 10 m*M* silver hydrogel. This shift in peak position indicates that the nanoparticles in the 10 m*M* silver hydrogel were smaller than those in the 5 m*M* silver hydrogel. TEM showed that the sizes of silver nanoparticles ranged from 2 to 16 nm.

The physical properties of the hydrogels were characterized including gel fraction, swelling, moisture retention, and WVTR. There were no significant differences between the neat hydrogel and silver nanoparticle infused hydrogels, indicating that the silver nanoparticles loaded in the hydrogels did not affect any physical properties of the AMPS-Na^+ hydrogels. The WVTR values ranged from 94.0 to 107.0 g $m^{-2}h^{-1}$ confirming that all hydrogels can be used to control body fluid loss and maintain a moist environment for burn wounds. Similarly, the tensile strength and % elongation results showed that the silver nanoparticles loaded in the hydrogels did not affect the mechanical properties of the neat hydrogel. It could be considered that all of the hydrogels have high tensile strength and percentage of elongation values. These properties support the use of these wound dressings as robust dressings.

The indirect cytotoxicity test results showed that none of the hydrogels were toxic to L929 and NHDF cells as the cell viability percentages were >90%. The cumulative release of silver was divided into two stages; initially there was a rapid release of 50%–70% within 24 hr of immersion, the second terminal stage was a slight but constant release after 72 hr of immersion. It can be concluded that the hydrogels could be used as potential silver release wound dressings for up to 72 hr.

The antibacterial activities against gram-positive *S. aureus* and gram-positive MRSA as well as gram-negative *P. aeruginosa* showed that the 5 m*M* silver hydrogel had the most inhibitory

activity for all bacterial growth. Sterilization at 121°C did not affect the antibacterial activity of the hydrogel. The 5 m*M* silver hydrogel provided similar effective antibacterial activities against gram-positive MRSA and *S. aureus*, with log reduction >3 after 6 hr of treatment. In the case of gram-negative *P. aeruginosa*, the results showed faster inhibition as the log reduction was >3 within 3 hr.

There are two explanations as why gram-positive bacteria are less susceptible to silver ions than gram-negative bacteria. Firstly, silver ions are positively charged so more silver may get trapped by the negative charge of the peptidoglycan cell wall in gram-positive bacteria. Secondly, the decreased susceptibility of gram-positive bacteria can also be explained by the fact that the cell wall of gram-positive bacteria is thicker than that of gram-negative bacteria.

Bacterial viability results showed that 5 m*M* silver hydrogel reduced the number of colonies to $<10^5$ CFU mL^{-1} after 3 hr of treatment for all three bacteria and the number of colonies was continuously reduced after this time. The silver hydrogel reduced all tested bacterial inocula to below detection limit within 12 hr.

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