Processing and Characterization of Antibacterial Hydrogel Sheet Dressings Composed of Poly(vinyl alcohol) and Silk Fibroin for Wound Healing Application[†]

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Abstract

Hydrogels are soft materials that contain high water content within their 3-dimensional structure. Such extremely hydrated environment allows hydrogels to recapitulate the structure of many native tissues inside the body. In biomedical application, hydrogels have been extensively used as biocompatible materials, drug delivery systems, and tissue-engineered scaffolds that can be designed to possess either permanent or slow-degradation properties. In this research, we applied gamma irradiation to develop transparent and conformal hydrogel sheets with sufficient mechanical strength from poly(vinyl alcohol) (PVA) and further modified the based PVA matrix with naturally-derived silk fibroin (SF) protein and silver nitrate ($AgNO_3$) for wound healing purpose. The physical and mechanical properties of based PVA hydrogels formed at varied irradiation doses from 10 - 80 kGy were first characterized. The dose of 60 kGy was found to be optimal to process flexible and elastic PVA sheets with equilibrium degree of swelling of 1000 %, gel fraction of 90 %, and tensile strength of 19 kPa. To further enhance water absorption capacity, 10 - 40 % (w/w) silk fibroin was added to the based PVA matrix. Based on water absorption and gel fraction data, hydrogel sheets with 8PVA:2SF formulation was selected for antibacterial test. Disc diffusion assay showed that the incorporation of 0.4 mM $AgNO_3$ in 8PVA:2SF hydrogel sheets could inhibit the growth of *Staphyllococcus aureus* and *Pseudomonas aeruginosa*. These results demonstrated that a prototypic, antibacterial hydrogel sheet dressing composed of both synthetic and natural polymers could be developed within a single-step by gamma irradiation technique.

Keywords: Hydrogel sheet dressing, PVA, silk fibroin, gamma irradiation, silver nitrate

Introduction

Hydrogels are 2 component systems consisting of 3-dimensional (3D) networks of hydrophillic polymers and water that fills the space between the polymer chains [1,2]. The high water content of hydrogels gives the materials advantages in biomedical application as it contributes to biocompatible property and minimal tendency to cause irritation [3-5]. Due to the ability to imbibe and retain a lot of water within their matrices without compromising structural integrity, hydrogels have been extensively used as contact lens [1,6-8], drug delivery systems, wound dressings [1,6,8-13], and tissue-engineered scaffolds [3-5,14-17].

Hydrogels can be formed by means of physical or chemical methods [1,10]. As for the second approach, ionizing radiation has long been recognized as a suitable tool for the formation of hydrogels [1,18-20] that possess stable, crosslinked networks of polymer chains in the 3D structure. The technique offers an ease of processing control over the use of harmful chemical initiators or crosslinkers, and also an ability to combine sterilization and joining of polymer chains within a single step. Synthetic polymers such as poly(vinyl alcohol) (PVA), poly(vinyl pyrrolidone) (PVP), poly(ethylene oxide), polyacrylamide, poly(acrylic acid), and poly(vinyl methyl ether) have been extensively investigated in the formation of hydrogels by both gamma and electron beam irradiation [1,6,8,13,20-23]. In addition, natural polymers such as chitosan [2,22,24], starch [8,13], carboxy methylcellulose [25], silk [9,16,26,27], agar [12,21,28], and carrageenan [6,23] are also added to PVA- and PVP-based solutions before the blended systems are rendered crosslinked by irradiation to improve the physical and mechanical properties of the resulting hydrogels. Examples of commercial hydrogel sheet dressings fabricated by irradiation techniques are KIK-gel®, Aqua-gel®, NuGel® and HiZel® [6,10].

Among various naturally-derived polymers used in the formation of hydrogel, silk fibroin (SF) has a long track record in medicine as suture [29] as well as its excellent mechanical and biocompatible properties [29,30] that makes SF as an attractive candidate in biomedical research. Many studies reported the use of silk fibroin in wound dressings [31-34], artificial skin substitutes [26], synthetic vascular grafts [30,35], drug delivery system [36,37], and tissue-engineered scaffolds [16,27,30]. For wound healing application, *in vivo* studies showed that SF-based dressings could promote the healing of full-thickness wounds with minimal inflammatory response in rat and mice models [31,33]. Among various fabrication techniques, gamma irradiation is utilized in the processing of SF and SF-blended PVA hydrogels for tissue-engineered scaffold [16] and artificial skin substitute [26], respectively. The SF hydrogels formed by irradiation-induced crosslinking showed an increase in mechanical strength to withstand compressive load compared to those prepared by physical entanglement of SF, and also were cytocompatible with human mesenchymal stem cells [16]. In addition, the complete transformation of random coils to beta-sheets in the SF structure was reported in SF-blended PVA hydrogels at elevated irradiation doses up to 40 and 50 kGy [26].

Materials and methods

Chemicals and reagents

All chemicals including PVA (M_w 89,000 - 98,000), calcium chloride ($CaCl_2$), sodium carbonate (Na_2CO_3), and silver nitrate ($AgNO_3$) were purchased from Sigma Aldrich (St. Louise, USA). Cocoons of Thai silkworms *B. mori* (variant Nangnoi Si Sa Ket) were obtained from the Chiang Mai Sericulture Center (Chiang Mai, Thailand). Silk fibroin protein was extracted from the as-received cocoons using the previously described method [39]. Reversed osmosis (RO) water was used in all experiments.

Preparation of PVA and SF-PVA hydrogel sheets

A solution of 10 % (w/v) PVA was prepared by dissolving PVA powder in hot water for 30 min and then mixed thoroughly by magnetic stirrer for 1 h. After the solution was cooled to room temperature (RT), it was deaerated with N_2 gas for 30 min before being transferred to 100×100 mm^2 square petri dishes. Each sample was sealed inside a plastic bag and then exposed to gamma irradiation at doses of 10, 25, 40, 60 and 80 kGy at the Gem Irradiation Center facility, Thailand Institute of Nuclear Technology (Public Organization). As a control, the starting PVA solution was left to form dried films inside the petri dishes at RT.

For the formation of PVA/SF hydrogel sheets, lyophilized SF protein was added to PVA solution at varied concentrations from 10 - 40 wt% while the PVA content in the final solution was made up to 100 wt%. After thorough mixing, the solution was transferred to circular 50-mm diameter molds and crosslinked by gamma irradiation.

Water absorption properties of hydrogels

After the irradiation process, hydrogel samples were washed 3 times, 10 min each, with RO water on a rotator at 60 rpm to remove unreacted molecules before being cut into 3×3 cm^2 samples. To simulate

the ability of gel sheets to absorb exudate at wound site (adapted from European Standard EN 13726), the samples were immersed in an aqueous solution containing 145 mmol NaCl and 2.5 mmol $CaCl_2$ (solution A). The increase in water uptake was calculated by the following equation.

$$\text{water uptake ratio} = W_s/W_i \tag{1}$$

where W_s was the swollen weight and W_i was the initial weight of hydrogels.

For swelling kinetics of hydrogels, selected samples were immersed in solution A for the entire duration of 72 h, and the swollen weight was recorded at 5, 15, 30 and 60 min, and then at 72 h. After the termination of water absorption at specified time point, all samples were dried to a constant weight at 60 ºC. Then the equilibrium degree of swelling (EDS) was calculated as;

$$\text{EDS (\%)} = W_s/W_d \tag{2}$$

where W_s was the swollen weight and W_d was the dried weight.

Gel fraction
Hydrogel samples were placed in glass tubes filled with RO water and boiled for 15 min to extract the loose, non-crosslinked polymeric molecules. The remaining gel portion was dried to a constant weight at 60 ºC. Gel fraction was then measured gravimetrically according to the following equation;

$$\text{Gel fraction (\%)} = W_f/W_o \tag{3}$$

where W_f was the final dried weight and W_o was the initial dried weight.

Mechanical properties of hydrogels
To estimate the mechanical properties of crosslinked hydrogels, the irradiated samples were examined by both tensile and compression tests. Tensile strength and elongation at break were measured by Lloyd LS1 (Ametek (GB) Ltd, UK) on rectangular gel samples (1×4 cm^2) at a crosshead speed of 50 mm/min.

For the analysis of gel strength, samples were prepared into disc shape with diameter 35 or 50 mm, and thickness of ~3 - 4 mm. All hydrogel samples were kept in hydrated environment until ready to be used. The samples were placed between a parallel plate apparatus and tested under uniaxial compression on Lloyd LS1 using a 50 N load cell that was set to compress the samples to 70 % gel thickness at a speed of 5 mm/min.

FTIR analysis of SF-PVA hydrogels
The PVA/SF hydrogels were lyophilized and pulverized. Infrared spectra of the gel samples were collected by attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy (Tensor 27, Brucker Optics, Germany) under absorbance mode from 4000 - 400 cm^{-1} at 4 cm^{-1} scan resolution. The starting PVA and silk fibroin powder was used as control.

Disc diffusion assay
Selected PVA/SF formulation was further incorporated with 0.4 - 2.0 mM $AgNO_3$ during the irradiation process. The resulting hydrogel sheets were cut into 8 mm diameter discs and placed onto Mueller-Hinton agar plates inoculated with 10^6 colony forming unit (CFU)/ml of either *S. aureus* or *P. aeruginosa*. After 18 h incubation at 37 ºC, digital images of the inhibited areas of bacterial growth surrounding each sample were recorded and measured.

Data analysis

For the analysis of water absorption, gel fraction, and tensile strength, data were collected from 3 different experiments. In terms of gel strength, data were collected from about 4 - 5 samples. For the antibacterial test, disc diffusion was performed in triplicates. Data were expressed as mean ± standard error of mean unless otherwise stated.

Results and discussion

Formation of based PVA hydrogel sheets

When a polymer solution is exposed to ionizing radiation, hydroxyl radicals (•OH) generated by radiolysis of water serves as the main reactive species that abstract hydrogen atoms off the polymer chains, thus initiating the joining or scission of these macromolecules [18,19]. During the joining process in which radicals on different chains recombine, the resulting intermolecular crosslinking can successfully contribute to the formation of 3D network that provides the structure of hydrogel. This usually requires the polymer concentration to be high enough above hydrodynamic concentration to enable the overlapping of polymer chains, which in turn increases the probability of intermolecular joining process [19]. In our PVA-based hydrogel system, the starting 10 % PVA solution could form gel with thicknesses ~2.0 - 2.5 mm after exposed to gamma irradiation as shown in **Figure 1**.

With the experimental conditions we had in this study, the irradiation doses of 40 and 60 kGy were found to be optimal for the formation of flexible and elastic hydrogels that were capable of retaining the flat sheet structure without being distorted. These PVA concentration and irradiation doses used in our system to fabricate hydrogel sheets were similar to previous studies [8,22] that reported the use of 10 % PVA solution to form hydrogels by e-beam irradiation at doses 40 - 50 kGy. At lower doses, the resulting sheets became too soft and easily rolled or folded into irregular shape. In particular, less than 20 % of the starting PVA solution was crosslinked and formed the sheet structure at 10 kGy. At the highest irradiation dose of 80 kGy, the hydrogel sheets lost their flexibility and were prone to break while being released off the mold. In contrast to these hydrogel sheets crosslinked by gamma irradiation, the as-cast PVA films formed at RT were shown to be rigid, stiff, and not pliable.
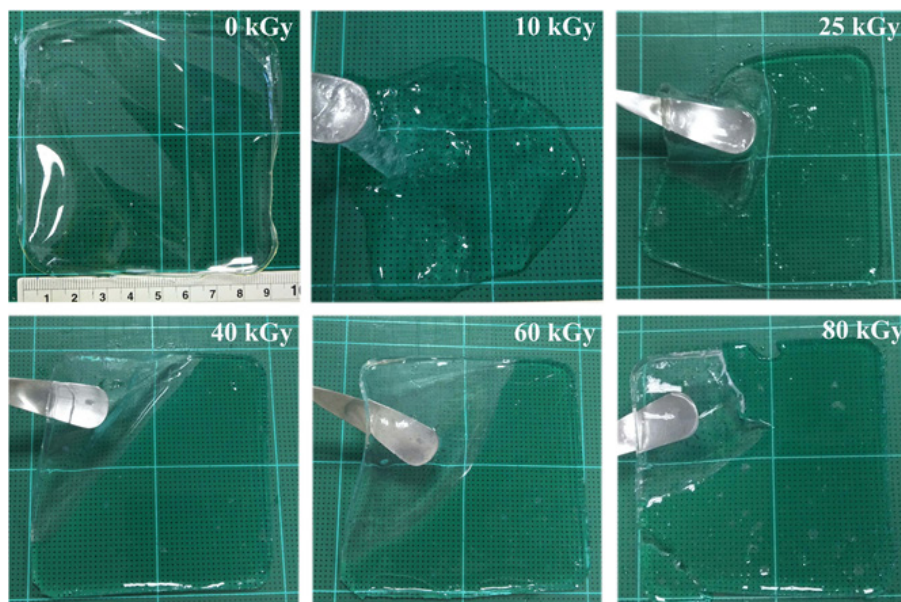
Figure 1 PVA hydrogel sheets formed at different irradiation doses from 10 - 80 kGy with as-cast, non-irradiated sheet (0 kGy) served as a control.

Water absorption ability of PVA hydrogels

The ability of hydrogel sheets to uptake additional water into their hydrated matrices was monitored as a function of time. As presented in **Figure 2**, PVA gel sheets crosslinked at 40 kGy showed a rapid increase in water uptake during the first hour of immersion in solution A before reaching a steady level after 24 h. The initial rise in swelling of hydrogel matrix can be attributed to the –OH functional group on PVA and spaces enclosed by crosslinked network that can attract and accommodate water molecules. As the elastic retraction force of the 3D network opposes, swelling eventually reaches equilibrium [10]. Therefore, the 24 h time point was chosen as the saturation, or equilibrium, point of swelling in our study based on this kinetics data. This time point was also within the same range compared to other hydrogel systems previously reported in the literature [40,44,45].
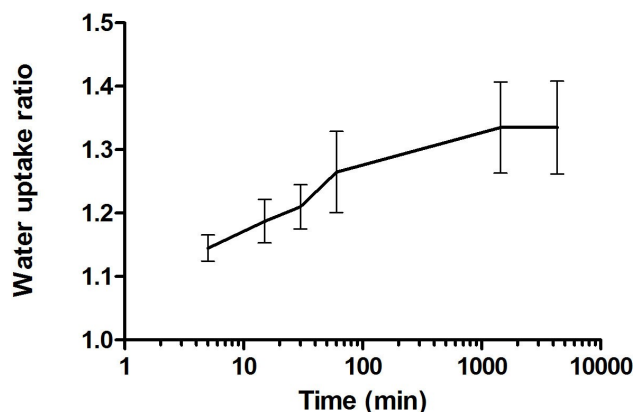
Figure 2. The swelling kinetics of crosslinked 10 % PVA hydrogel sheets formed at 40 kGy.

The equilibrium degree of swelling of hydrogel sheets prepared at different irradiation doses is shown in **Figure 3A**. As the doses were raised from 25 - 80 kGy, the swelling of hydrogel sheets decreased from ~2,200 to 1,000 % before reaching a steady level at doses ≥ 40 kGy. This reduction in water absorption observed in our system is in agreement with other PVA and PVP systems previously reported [40,45]. The smaller mesh size of 3D network to accommodate water molecules caused by the increase in crosslink density can be responsible for the reduction in water absorption at high doses.
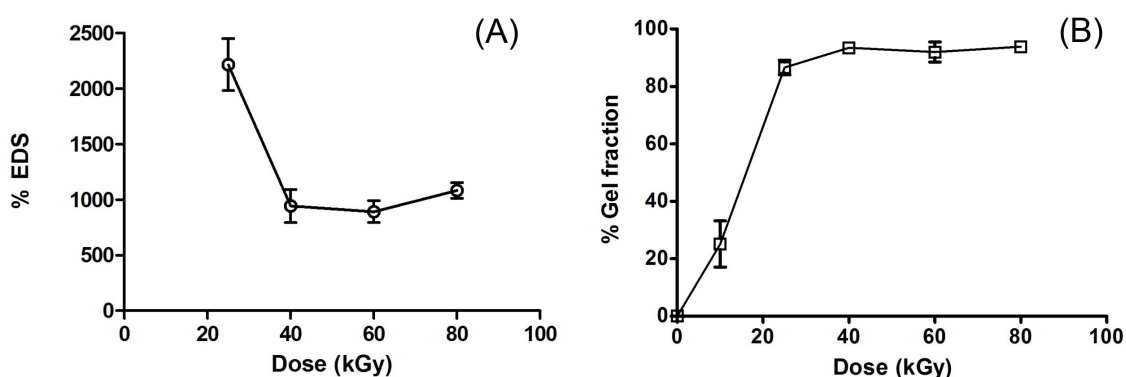
Figure 3 (A) Swelling behavior and (B) gel fraction of hydrogel sheets formed at varied irradiation doses.

Gel fraction

The content of stable 3D network formed by radiation-induced crosslinking was characterized and represented by gel fraction as shown in **Figure 3B**. In contrast to swelling, gel fraction increased with irradiation doses from 10 to 40 kGy before reaching a steady level of ~90 %. This maximum extent of gel fraction of our PVA system obtained at irradiation dose $\geq$ 40 kGy is within the same range as other PVA-based hydrogels reported in the literature [8,22,40]. As a control, the as-cast PVA films did not contain any chemical crosslinked network within the structure and were dissolved in boiling water. Based on water absorption and gel fraction data, the structural characteristics of hydrogel network is strongly affected by crosslink density, which can be controlled by irradiation dose. In our system, the doses of 40 - 60 kGy seemed to be the optimal dose range that provided flexible and elastic hydrogel sheets with the highest degree of gel fraction.

Mechanical properties of PVA hydrogels

Gel strength was measured at 70 % gel depth and represented by compressive loads as shown in **Table 1**. The ability of hydrogels to withstand compressive load continued to increase with irradiation dose from 25 - 60 kGy. At the highest dose of 80 kGy, gel strength decreased slightly, which could be caused by degradation of PVA molecules that prevailed crosslinking. This lowered gel strength was also in agreement with our observation shown in **Figure 1**.

Table 1 Mechanical properties of hydrogels formed at varied irradiation doses.

Dose (kGy)	Compressive load (N)	Tensile strength (kPa)	Elongation at break (%)	Young's modulus (kPa)
25	3.4 ± 0.8	n/a	n/a	n/a
40	12.4 ± 2.0	8.2 ± 1.9	89.5 ± 23.1	7.7 ± 3.4
60	18.0 ± 2.4	18.8 ± 4.7	128.4 ± 33.8	10.2 ± 3.4
80	12.6 ± 0.8	19.4 ± 4.3	79.3 ± 30.2	23.1 ± 10.0

For the analysis of tensile strength, only the gel sheets formed at 40 - 80 kGy were tested under tension as the sheets formed at 25 kGy were too soft and easily torn during the test setup. Similar to gel strength, tensile strength and elongation at break increased as irradiation dose was raised from 40 to 60 kGy. At 80 kGy, the elongation at break was largely reduced. Based on these data obtained from both tensile and compression tests as well as water absorption and gel fraction, the dose of 60 kGy seemed to be best for the preparation of hydrogel sheets with optimal physical properties and mechanical strength. Therefore, we selected the dose of 60 kGy to further fabricate PVA/SF hydrogels.

Structural characteristics of PVA-SF hydrogels

Various natural polymers, especially polysaccharides [6,8,22,23,45], have been used to blend with synthetic polymers to improve both physical and mechanical properties of the resulting irradiated hydrogels. In this study, we chose SF protein extracted from the cocoons of silkworm to blend with based PVA hydrogel system. Changes in water absorption properties and content of stable 3D crosslinked network were characterized by water uptake ratio and gel fraction as shown in **Figure 4**.
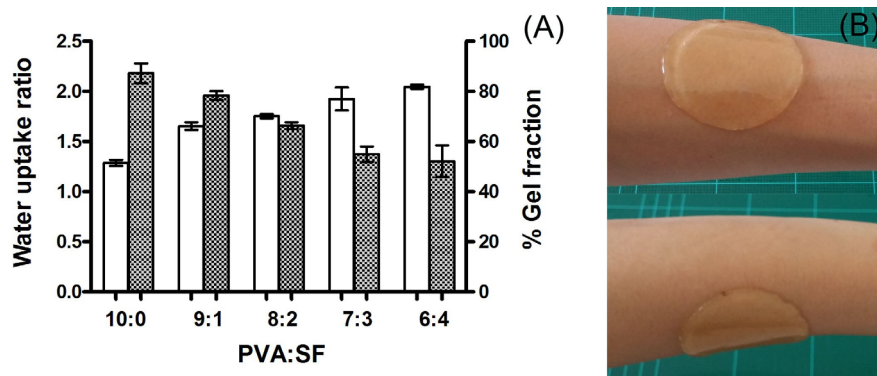
Figure 4 (A) Swelling behavior (solid white) and gel fraction (patterned gray) of blended PVA/SF hydrogel systems at varied SF proportion from 0 - 4 % (w/w). (B) The hydrating, flexible and conformal blended hydrogel sheet fabricated from 8PVA:2SF formulation at 60 kGy.

With the increase in SF proportion from 10 - 40 % (w/w), the ability of blended PVA-SF hydrogels to uptake additional water was enhanced from 1.3 to 2.0, whereas the content of stable crosslinked network decreased by ~20 %. Thus, the main crosslinked network is largely contributed by PVA molecules, while SF molecules may be grafted on PVA chains, similar to the PVA-chitosan blend system [22]. So functional groups such as -COOH and -NH$_2$ of the side chains on SF molecules can attract additional water molecules. In addition, the proportion of SF beyond 20 % (w/w) also made gel sheets to become too soft, and easily folded. Therefore, the 8PVA:2SF blend system was the optimal formulation that provided a flexible and conformal gel sheet (**Figure 4B**) with improved water absorption property.

Chemical analysis of PVA/SF hydrogels

Figure 5 shows FTIR spectra of 8PVA:2SF hydrogel in comparison to the starting PVA and SF components. The vibration signals of O-H and C-H from alkyl groups of PVA could be seen at 3550 - 3200 and 3000 - 2840 cm^{-1}, respectively, similar to the findings on blended PVA hydrogel system previously reported in literature [46]. In addition, the N-H bending vibration intensity of β-sheets in SF protein [39] could also be detected at 1650 - 1610 and 1540 - 1500 cm^{-1}. These chemical signals confirmed the presence of both PVA and SF in the blended hydrogels after irradiation process.
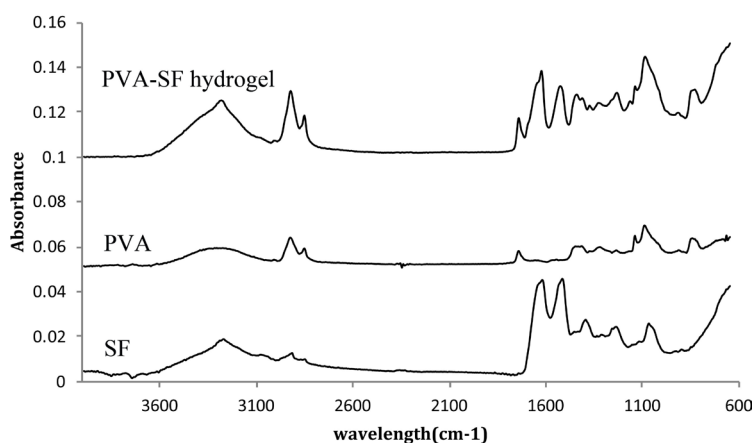

Figure 5 FTIR spectra of blended 8PVA:2SF hydrogel formed by irradiation compared to the starting PVA and SF powder.

Antibacterial properties

Irradiation of silver compound in aqueous solution during the formation of hydrogel was shown to induce the formation of small Ag^+ clusters that conferred hydrogel matrix with antibacterial properties [40]. The binding of these Ag^+ clusters to proteins and DNA of bacteria leads to the disruption of cell and organelle membranes as well as DNA that eventually result in cell death [38,39,41]. In our study, we used disc diffusion assay to evaluate the antibacterial properties of 8PVA:2SF hydrogel sheets incorporated with $AgNO_3$. Our results showed that the minimal concentration of $AgNO_3$ at 0.4 mM could inhibit the growth of both *S. aureus* and *P. aeruginosa* as evidenced by the formation of a clear zone as shown in **Figure 6**. This concentration was within the same range of what has been previously reported [39]. In addition, the inhibition ratios [39], defined as the diameter of the clear zone normalized by the diameter of hydrogel, was calculated to be 2.15±0.19 for *S. aureus* inoculated plates and 1.35±0.04 for *P. aeruginosa* plates. These inhibition ratios we obtained in the present study followed the similar trend with values previously reported [39] as the clear zones around hydrogel samples on *S. aureus* inoculated plates were larger than those on *aeruginosa* plates. In addition, the diameter of clear zone, ~10 mm, formed around our AgNP-embedded hydrogel, 7.5 mm, against *P. aeruginosa* was within the same range as the values obtained from commercial dressings and also other hydrogel systems containing AgNPs [43].
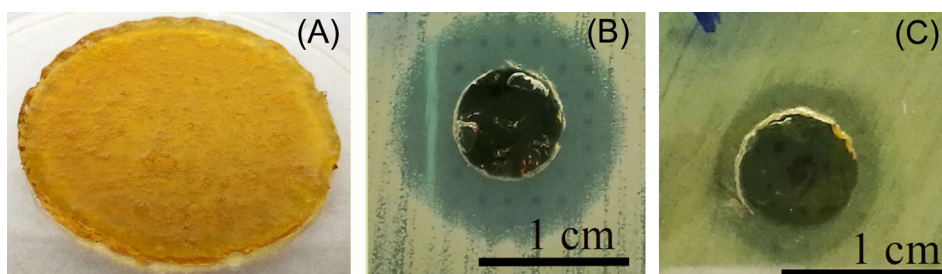
Figure 6 Digital images showing (A) 8PVA:2SF hydrogel sheet incorporated with 0.4 mM $AgNO_3$, and the formation of clear zone on agar plates inoculated with (B) *S. aureus* and (C) *P. aeruginosa*.

Conclusions

In this study, we have demonstrated the feasibility of applying gamma radiation to fabricate hydrogel sheet dressings composed of multiple components as well as active ingredient in a single-step method. To maintain the mechanical strength of sheet dressings, PVA was needed as the major based component, whereas naturally-derived SF could be further added at the maximum of 20 % weight content to enhance water absorption property of hydrogel sheets. The incorporation of antiseptic $AgNO_3$ into PVA/SF blend during irradiation could effectively confer the sheet with antibacterial properties. Therefore, this prototypic antibacterial hydrogel sheet dressing can be further developed for the potential use in clinics.

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