Sericin Ameliorates the Properties of Poly(Vinyl Alcohol) Hydrogel Prepared by Simple Repeated Freeze-Thaw Process without the Use of Chemical Crosslinking

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***Abstract*—Hydrogel of polyvinyl alcohol (PVA) and sericin can be easily produced using a repeated freeze-thaw process. The effects of polymer concentration) 4-8 %wt(, blending ratio of PVA/ sericin) 100/ 0-50/ 50(, and the number of freeze-thawing cycle)4, 8, and 12 cycles(on chemical and physical properties of the hydrogels obtained were studied. We here showed that higher polymer concentration, higher PVA ratio, and more cycles of freeze-thawing produced the hydrogels with high gel (crosslinked(fraction (>90), wall-like structure, and high compressive modulus (100-170 kPa). When the sericin ratio was increased, the hydrogels showed less gel fraction) 60-80(, more porous structure with highly interconnected pores, and better swelling ability (up to 8-9 times of its original state(. The formation of the PVA/ sericin hydrogels was occurred by the conformational changes of both PVA and sericin. The secondary structures of PVA and sericin turned to more stable crystalline conformation during the freeze-thawing process, as confirmed by fourier transform infrared (FTIR(spectroscopic results. Furthermore, all hydrogels were not toxic to human skin keratinocytes (HaCaT) cells while the anti-oxidant activity of sericin component in hydrogels was confirmed. We concluded that the freeze-thawing process was a simple and effective technique for fabrication of PVA and sericin, which both are water-soluble, into the stable hydrogels without the use of any chemical solvents or further crosslinking. More importantly, sericin enhances the biological activities of the hydrogels, allowing the use of this hydrogel in various medical applications such as wound dressing.**

***Index Terms*— freeze-thaw; sericin; PVA; hydrogel; conformation; anti-oxidant.**

I. Introduction

The poly(vinyl alcohol)/sericin hybrids have been introduced as materials for tissue engineering, wound dressing, drug delivery system in various formulations e.g. films [1] scaffold[2,3] hydrogel[4], or electrospun nanofiber[5]. Poly (vinyl alcohol) (PVA) is a linear synthetic polymer which forms copolymers of vinyl alcohol and vinyl acetate. PVA is widely used in medical applications because it is water soluble and biodegradable while induces minimal immunological response[6]. However, PVA itself does not have biological activities. On the other hand, proteins such as sericin derived from the silkworm cocoon naturally have various biological functions, for example, anti-bacteria, anti-oxidation, anti-inflammation, bio-adhesion, moisturizing ability, and mitogenic effect on mammalian cells[7]. Blending sericin with PVA was reported to improve cell attachment and proliferation, collagen formation, epithelialization, and wound healing efficiency of the material[8-9]. To avoid using chemical crosslinking agents, Yamdej et al. applied gamma irradiation to physically crosslink and form the PVA/sericin hydrogels having high water content[10]. The gamma could either form or sterilize the hydrogels at the same time, thus further sterilization is not required. However, the limitations of gamma irradiation are high cost of a gamma irradiator, difficulty to control the degree of crosslinking, and the degradation of some polymer when exposing to gamma radiation.

In this study, we exhibited the new method for fabrication of the PVA/sericin hydrogels by using the repeated freeze-thawing cycle which is a very simple technique without the use of solvents or any crosslinking agents. PVA hydrogel has been fabricated using this technique as previously report, however, its rigidity and non-biological advantage in wound healing are the main concern[11-14]. Herein, we introduced the adding of sericin into PVA hydrogel during fabrication to improve the flexibility and biological activities of the hydrogels. The various concentrations and blending ratios of the PVA/sericin were varied for the fabrication. The effects of polymer concentration, blending ratios and freeze-thawing cycles on the properties of PVA/sericin hydrogels including gel fraction,

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morphology, mechanical properties, and the release of sericin were systematically investigated. The chemical structures were also analyzed by fourier transform infrared (FTIR) spectroscopy to investigate the mechanism of hydrogel formation. In addition, in vitro cytotoxic and anti-oxidant activities of the hydrogels were elucidated.

II. Materials and Methods

A. Materials

White-shell Bombyx mori silk cocoons were kindly supplied by Chul Thai Silk Co., Ltd., Petchaboon province, Thailand. Polyvinyl alcohol (PVA, molecular weight 115000, degree of polymerisation 1700-1800, viscosity 25-32 cp, hydrolysis 98-99 %mol) was purchased from LOBA Chemie, India. Glycerin and other chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA) and used without further purification.

B. Extraction of sericin

The silk cocoons were cut into small pieces and the sericin was extracted using a high temperature and pressure degumming technique (1 g cocoon per 15 g water), as reported previously[15]. A silk sericin solution was obtained as measured using the BCA Protein Assay Reagent (Pierce, Rockford, IL, USA). The sericin solution was concentrated to 10% w/v by a solvent evaporation technique using heat treatment.

C. Preparation of PVA/sericin hydrogels using freeze-thawing technique

Polyvinyl alcohol was dissolved in deionized (DI) water at 80°C for 4 h to prepare a PVA solution. Sericin solution was mixed with PVA solution at different weight blending ratios (PVA/SS: 100/0, 90/10, 80/20, 70/30, 60/40, and 50/50). The final concentration of the mixed PVA/SS solution was varied at 4, 5, 6, 7, and 8 %wt. The mixed solution was poured into aluminum mold and frozen at -20°C for 48 h. Then, the frozen hydrogel was thawed at 25°C for 24 h. This step was called as 1 cycle of freeze-thaw. The number of freeze-thaw cycles was varied at 4, 8, and 12.

D. Evaluation of gel fraction of the freeze-thawed PVA/sericin hydrogels

Gel fraction of the hydrogels was evaluated following the method reported by Kamoun et al[16]. Briefly, the hydrogels (1 ×1×1 cm^3) were dried in an oven at 50°C for 24 h. The dry hydrogel was weighed (W_o) and immersed in deionized water for 24 h. The non-crosslinked fraction of the hydrogel was solubilized in this step. Then, the remained hydrogels were dried again and weighed (W_t). The gel fraction percentage of the hydrogels was calculated according to the equation 1 :

$$\% \text{ Gel fraction} = W_t / W_o \times 100 \quad (1)$$

E. Morphological observation

The freeze-thawed hydrogels were freeze-dried using lyophilizer. The cross-sectioned morphology of the dried hydrogels was observed on a scanning electron microscope (SEM, JSM 5400, JEOL) at an accelerating voltage of 12–15 kV after sputter-coating with gold.

F. Mechanical test of the freeze-thawed PVA/sericin hydrogels

A compression and tensile tests were performed on the hydrogels (15×15×2 mm^3) at room temperature using a Universal Testing Machine (Hounsfield H10KM, UK) equipped with a 10-kN load cell at a constant rate of 30 mm/min. The curves of force as a function of deformation (mm) were automatically recorded by the software. The compressive and tensile modulus of the hydrogels were calculated according to the ASTM D638-01 method (n = 6).

G. Fourier transform infrared spectroscopic analysis

The functional groups in the PVA/sericin hydrogels were examined using Fourier transform infrared (FT-IR) spectroscopy (Perkin-Elmer, USA). The information on structural contributions was collected in the FT-IR analysis using Perkin-Elmer Spectrum GX (FT-IR system). The FT-IR analysis was based on the identification of absorption bands attributed to the vibrations of functional groups present in the samples. All spectra were recorded in the wavenumber range from 4000 to 400 cm^{-1} at a resolution of 4 cm^{-1}. Subsequently, the fourier self-deconvolution along with peak fitting of the amide I region (1600−1700 cm^{-1}) was analyzed to evaluate the secondary structure of sericin, according to the procedure reported by Hu et al[17].

H. In vitro release of sericin from the freeze-thawed PVA/sericin hydrogels

The hydrogels (1×1×1 cm^3) were placed into 10 mL of phosphate-buffered saline solution (PBS, pH 7.4) at 37°C with continuous stirring at 100 rpm in a closed container. Aliquots of the PBS solution (1.5 mL) were collected at the predetermined times, and the amount of sericin released into the solution was measured using a BCA protein assay kit as described previously (n = 3).

I. Cytotoxicity Test

Human skin keratinocytes (HaCaT) were precultured for 24 h in 96-well plates (10,000 cells per well) in DMEM containing antibiotic solution (100U/mL of penicillin and 100ug/mL of streptomycin) and 10% FBS (Gibco). On the other hand, the hydrogels were soaked in DMEM for 24 h to prepare hydrogels'extracted medium. Then, the medium of cell culture was replaced with the hydrogels'extracted medium and cultured for further 24 h. Cell viability was evaluated using Presto Blue™ metabolic assay. The fluorescent intensity was measured at wavelengths 560 nm excitation and 590 nm emission using microplate reader (Thermo, Varioskan Flash, England).

J. Oxidative Stress Assessment

To determine the free radicals and reactive oxygen species (ROS). HaCaT cells were seeded on 96 well black plated (10,000 cells per well) for 24 h. Cells were incubated with the DCFHDA (molecular probe, USA) for 30 min. At the end of

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this period, medium was removed and cells exposed to the H_2O_2 (0.8mM) mixed with sericin or sericin released in medium (hydrogels were soaked in DMEM for 6 h, 1 day, 2 day, 3 day, 5 day and 7 day). After incubation of cells exposed to samples at 37°C for 1 h, the fluorescent intensity was measured for each time period at 485 nm (excitation) and 527 nm (emission) wavelengths using a microplate reader (Thermo, Varioskan Flash, England).

K. Statistical Analysis

All quantitative data were shown as mean ± SD. The statistical significance was determined by paired and unpaired Student's t-tests along with ANOVA. A value of $p < 0.05$ was considered to be significant.

III. Results

A. Gel fraction of the freeze-thawed PVA/sericin hydrogels

Figure 1 shows the gel fraction of different formulations of PVA/sericin hydrogels fabricated by freeze-thaw technique for 4, 8, and 12 cycles. For the PVA and PVA/SS 80/20 hydrogels fabricated at 4-8%wt, the gel fraction seemed slightly increased with the increasing concentration. This effect was more clear when the hydrogels were freeze-thawed for only 4 cycles. The PVA hydrogels had slightly higher gel fraction than the PVA/SS 80/20 hydrogels. Considering the 8%wt hydrogels with different PVA/SS blending ratios, it was found that th gel fraction was significantly reduced when the ratio of sericin was increased. Comparing the cycles of freeze-thaw, the more freeze-thawing cycle formed the hydrogels with a significant higher gel fraction than the less freeze-thawing cycle.

B. Morphology of the freeze-thawed PVA/sericin hydrogels

The cross-sectioned morphology of different formulations of PVA/sericin hydrogels fabricated by freeze-thaw technique for 4, 8, and 12 cycles was demonstrated in Fig. 2 PVA hydrogels showed wall-like structure while the mixed PVA/sericin hydrogels had more porous structure with interconnected pores. The hydrogels with more sericin ratio seemed to have smaller pore size. The number of freeze-thaw cycle did not show significant effect on morphology of the hydrogels.

C. Mechanical properties of the freeze-thawed PVA/sericin hydrogels

Figure 3(A) and 3(B) show the compressive and tensile modulus, respectively, of different formulations of 8%wt PVA/sericin hydrogels fabricated by freeze-thaw technique for 4, 8, and 12 cycles. The highest compressive

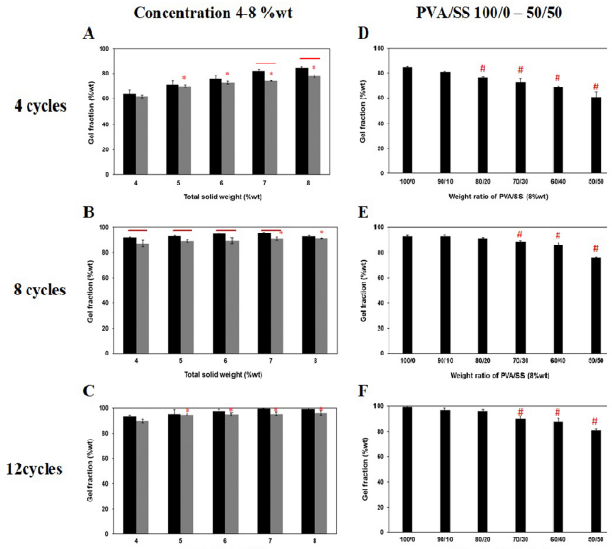


Fig 1. Gel fraction of the PVA/SS hydrogels fabricated by freeze-thawing process for 4, 8, and 12 cycles, (A-C) PVA (■) and PVA/SS 80/20 (■) hydrogels, (D-F) Different weight blending ratios of PVA/SS of 100/0 - 50/50. * represent a significant difference at $p < 0.05$ when compared to the values of 4% wt .# represent a significant difference at $p < 0.05$ when compared to the value of 100/0 hydrogel.

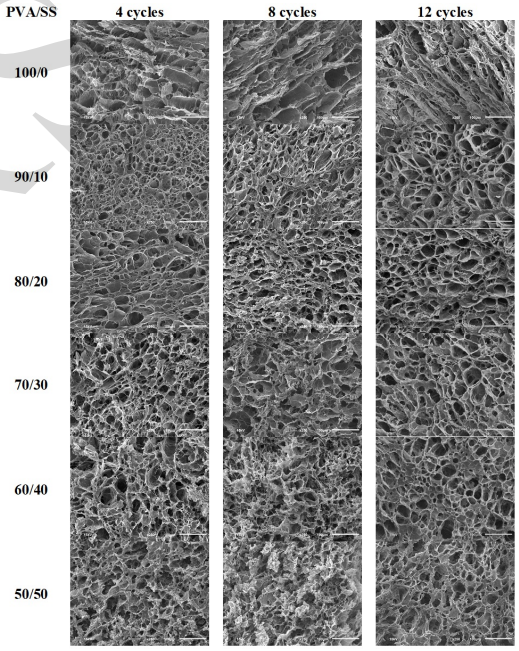


Fig 2. SEM images of the PVA/SS hydrogels fabricated by freeze-thawing process for 4, 8, and 12 cycles. The PVA/SS hydrogels have at total solid weight concentrations of 8%wt and different weight blending ratios of PVA/SS of 100/0 - 50/50.

modulus was found in PVA hydrogels while it seemed to decrease with the increasing ratio of sericin. It was also observed that the more cycles of freeze-thawing process were likely to improve the compressive modulus of the hydrogels

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Considering tensile modulus, the significant difference among hydrogel formulations was not noticed. On the other hand, the more cycles of freeze-thawing process statistically enhanced the tensile modulus of the hydrogels.

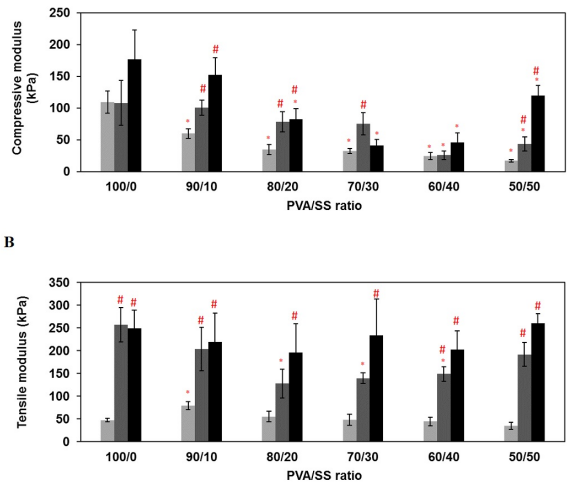


Fig 3. (A) Compressive modulus and (B) Tensile modulus of the PVA/SS hydrogels fabricated by freeze-thawing process for 4 (), 8 (), and 12 cycles (). The PVA/SS hydrogels have at total solid weight concentrations of 8%wt and different weight blending ratios of PVA/SS of 100/0 - 50/50. * represent a significant difference at $p < 0.05$ when compared to the value of 100/0 hydrogel. # represent a significant difference at $p < 0.05$ when compared to the value of 4 cycles freeze-thawing process.

D. Chemical structure of the freeze-thawed PVA/sericin hydrogels

The FTIR spectra of the sericin, PVA, and the mixed PVA/sericin (50/50) hydrogels fabricated by freeze thaw step at 4, 8, and 12 cycles were shown in Figure 4. A broad peak of O–H vibration at 3341 cm^{-1} was found in the spectrum of all samples. The vibration of a C–H and C–O bonds was clearly observed at 2940-2924cm^{-1} and 1090–1093 cm^{-1}, respectively, for PVA alone and PVA/sericin (50/50) hydrogels. The characteristic absorptions of protein, including amide I (1600-1700 cm^{-1}), amide II (1500-1560 cm^{-1}), and amide III (1200-1300 cm^{-1}), were markedly observed in the sericin sample, sericin freeze-thawed for 4 cycles, and PVA/sericin (50/50) hydrogels[18]. After deconvolution of amide I region, the percentage of secondary structure of sericin in the mixed PVA/sericin (50/50) hydrogels was analyzed, as presented in Table 1. It was obviously seen that the sericin when mixed with PVA and fabricated by freeze-thawing process had a significant higher percentage of β-sheet structure. Interestingly, the sericin freeze-thawed for 4 cycles without PVA had the similar percentage of β-sheet structure to the original sericin. This implied that the presence of PVA during freeze-thawing process strongly affect on secondary conformation of the sericin. The high β-sheet content of the freeze-thawed PVA/sericin hydrogels indicated their stable chemical structure.

E. In vitro release profiles of sericin

Figure 5 demonstrates the profiles of sericin released from different formulations of 8%wt PVA/sericin hydrogels

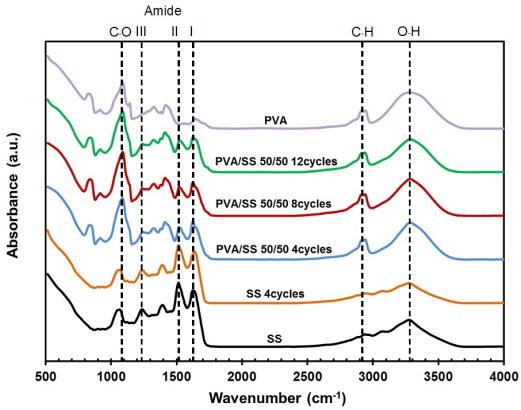


Fig 4. FTIR spectra of sericin, PVA, and the mixed PVA/sericin (50/50) hydrogels fabricated by freeze thaw step at 4, 8, and 12 cycles.

TABLE I. PERCENTAGE OF SECONDARY STRUCTURE OF SERICIN, PVA, AND THE MIXED PVA/SERICIN (50/50) HYDROGELS FABRICATED BY FREEZE THAW STEP AT 4, 8, AND 12 CYCLES, DECONVOLUTED FROM AMIDE I REGION OF FTIR SPECTRA.

Secondary structure	SS	SS 4cycles	PVA/SS 50/50 4cycles	PVA/SS 50/50 8cycles	PVA/SS 50/50 12cycles
Aggregated strands	14.02	5.27	0.00	0.00	0.00
Beta sheet	30.69	31.10	46.10	45.43	40.33
Random coil	22.30	26.44	21.56	19.24	25.52
Alpha-helices	10.27	10.55	9.47	11.84	9.78
Turns	22.72	26.63	22.87	23.50	24.37
total	100.00	100.00	100.00	100.00	100.00

fabricated by freeze-thaw technique for 12 cycles when incubated in PBS solution (pH 7.4) at 37°C. All hydrogels continuously released sericin along 168 h at which 70-90% sericin was released out. When comparing among various hydrogel formulations, the significant difference in release rate was not obvious. The hydrogels formed by 12 cycles of freeze-thawing process released ~70% sericin at 168 h.

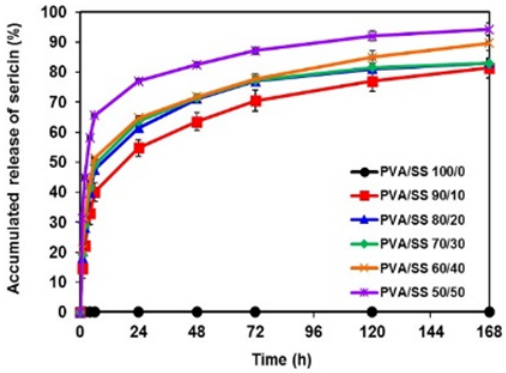


Fig 5. Percentage of accumulative release of sericin from the PVA/SS hydrogels fabricated by freeze-thawing process for 12 cycles when incubated in PBS solution (pH 7.4) at 37°C. The PVA/SS hydrogels have at total solid weight concentrations of 8%wt .

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F. Cell viability

Figure 6A shows viability percentage of HaCaT cells cultured with hydrogels' extracted medium for 24 h, comparing to those cultured in the presence of H_2O_2 as a positive control. The results demonstrated that all PVA/sericin hydrogels were not toxic to HaCaT cells. The cell viability was about 100%. On the other hand, the cells exposed to H_2O_2 remain viable only 10%.

G. Anti-oxidant activity

The percentages of ROS generation of HaCaT cells cultured with H_2O_2 in the presence of sericin or sericin released from hydrogels were shown in Figure 6B. The generation of ROS by cells treated with H_2O_2 was markedly increased to 530%. However, when the cells were treated with H_2O_2 in the presence of sericin (20-200 µg/mL), the ROS percentage was reduced significantly to around 300-390%, comparing to 100% of normal cells without H_2O_2 treatment. For the PVA/sericin (50/50) hydrogels, the sericin high released from hydrogels within the first 6 h. The ROS percentage was reduced significantly to 346 % while PVA/sericin (90/10) hydrogels seemed slightly decreased with the increasing times. The statistical difference in ROS generation among cells treated with sericin extracted from different hydrogels was not observed.

IV. Discussion

In this study, we reported the successful fabrication of the high water content (<90%) PVA/sericin hydrogels at various concentrations (4-8%wt) and weight blending ratios (100/0-50/50) by using a very simple repeated freeze-thawing process (4, 8, and 12 cycles). We showed that the properties of the obtained PVA/sericin hydrogels depended on the all parameters above (polymer concentration, weight blending ratio of PVA/sericin, and freeze-thaw cycles). The higher concentration of polymer increased the gel fraction of the hydrogels, implying the higher crosslinked fraction (Fig. 1). This can be explained that the high concentration contained more polymer molecules to interact each other, making the covalent bonds and the crosslinked molecules within its structure. The similar phenomenon was also found in case of gelatin hydrogels reported by Bukhari et al [19]. The gel fraction of gelatin hydrogels was increased along the increased gelatin concentration. Moreover, we found that the gel fraction (crosslinked fraction) of the hydrogels was influenced by the blending ratio of PVA/sericin. Although the increase in sericin ratio slightly reduced the crosslinking of hydrogels (Fig. 1) ,the sericin addition improve the flexibility of and softness of the hydrogels, as can be seen from the reduced compressive modulus (Fig. 3A). It was also found that the hydrogels with higher sericin ratio had better swelling ability due to the hydrophilic property of sericin [20] and their highly porous structure (Fig. 2).

In addition, the effects of freeze-thawing cycle on chemical and physical properties of the PVA/sericin hydrogels were herein elucidated. The more freeze-thawing cycles produced the highly crosslinked hydrogels (Fig. 1). This can be assumed that the repeated freeze-thawing cycles may alter the conformation of secondary structure of sericin within the hydrogels to be more stable that were confirmed by FTIR results (Fig. 4 and Tables 1). The freeze-thawed PVA/sericin hydrogels showed significantly higher percentages of β-sheet

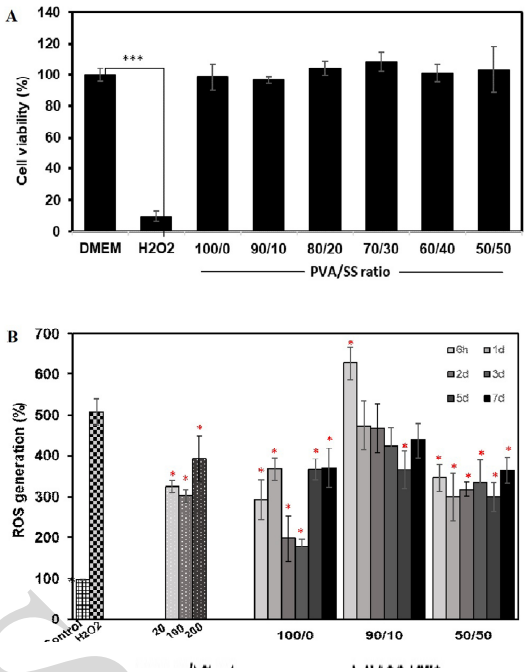


Fig 6. (A) Viability percentage of HaCaT cells cultured with extracted medium of PVA/sericin hydrogels for 24 h and (B) ROS generation of HaCaT cells cultured with H_2O_2 in the presence of sericin or sericin released in medium. *represent a significant difference at $p < 0.05$ when compared to the value of H_2O_2 group

conformation when comparing with the original sericin and PVA. It was interesting to notice that the presence of PVA during the freeze-thawing process strongly increased the β-sheet conformation because the freeze-thawed sericin alone showed similar structure to the original sericin. Taken together, either the presence of PVA or the repeated freeze-thawing cycles were the significant factors to produce the highly stable PVA/sericin hydrogels. The higher compressive and tensile modulus of the hydrogels freeze-thawed for more cycles further verified their stable chemical structure occurred (Fig. 3). The fabrication of PVA hydrogels by repeated freeze-thawing cycles has been previously reported by Hassan and Peppas. An increase in the initial concentration of aqueous PVA solutions resulted in the higher initial crystalline structure [21]. This phenomenon could be one of mechanisms to explain the gelation of the PVA/sericin blends of this study. Interestingly, we found that all blending ratios of PVA/sericin hydrogels gradually released the sericin in a similar rate (Fig. 5). The sustained release of sericin from the hydrogels would show some biological activities such as promoted cell attachment and proliferation, collagen formation, epithelialization, and wound healing acceleration, as previously reported [8,9].

The most advantage of our study was that the repeated freeze-thawing process could successfully form PVA and sericin, which both are water-soluble, into the stable hydrogels

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without the use of any chemical solvents or further crosslinking. The obtained hydrogels were not toxic to the cells (Fig. 6A) while the sericin in the hydrogels remained its anti-oxidant activity (Fig. 6B). Furthermore, this process is simple and does not require any sophisticated instruments while the good reproducibility is obtained. This repeated freeze-thawing process can be further applied to other polymers or blends which have similar conformational properties.

V. Conclusion

The repeated freeze-thawing process was introduced to fabricate the hydrogels of PVA/sericin. The polymer concentration, blending ratio of PVA/sericin, and the number of freeze-thawing cycle influenced on chemical and physical properties of the hydrogels obtained. The PVA/sericin hydrogels with higher polymer concentration and PVA ratio formed by more cycles of freeze-thawing showed the high gel (crosslinked) fraction, wall-like structure, and high compressive modulus. On the other hand, the hydrogels with the increased sericin ratio had less gel fraction, porous structure with highly interconnected pores, and good swelling ability. The formation of the PVA/sericin hydrogels was occurred by the conformational changes of both PVA and sericin. The secondary structures of PVA and sericin turned to more stable crystalline conformation during the freeze-thawing process. Furthermore, all PVA/sericin hydrogels were not toxic to HaCaT cells and the anti-oxidant activity of sericin in the hydrogels was confirmed. Therefore, we suggested the simple freeze-thawing process for the fabrication of the water-soluble PVA and sericin into the stable hydrogels without the use of any chemical solvents or further crosslinking.

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