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Research Article

Theme: Sterile Products: Advances and Challenges in Formulation, Manufacturing, Devices and Regulatory Aspects
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The Effect of Sterilization Methods on the Physical Properties of Silk Sericin Scaffolds

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Abstract. Protein-based biomaterials respond differently to sterilization methods. Since protein is a complex structure, heat, or irradiation may result in the loss of its physical or biological properties. Recent investigations have shown that sericin, a degumming silk protein, can be successfully formed into a 3-D scaffolds after mixing with other polymers which can be applied in skin tissue engineering. The objective of this study was to investigate the effectiveness of ethanol, ethylene oxide (EtO) and gamma irradiation on the sterilization of sericin scaffolds. The influence of these sterilization methods on the physical properties such as pore size, scaffold dimensions, swelling and mechanical properties, as well as the amount of sericin released from sericin/polyvinyl alcohol/glycerin scaffolds, were also investigated. Ethanol treatment was ineffective for sericin scaffold sterilization whereas gamma irradiation was the most effective technique for scaffold sterilization. Moreover, ethanol also caused significant changes in pore size resulting from shrinkage of the scaffold. Gamma-irradiated samples exhibited the highest swelling property, but they also lost the greatest amount of weight after immersion for 24 h compared with scaffolds obtained from other sterilization methods. The results of the maximum stress test and Young's modulus showed that gamma-irradiated and ethanol-treated scaffolds are more flexible than the EtO-treated and untreated scaffolds. The amount of sericin released, which was related to its collagen promoting effect, was highest from the gamma-irradiated scaffold. The results of this study indicate that gamma irradiation should have the greatest potential for sterilizing sericin scaffolds for skin tissue engineering.

KEY WORDS: ethanol; ethylene oxide; gamma irradiation; scaffold; sericin.

INTRODUCTION

Tissue engineering is widely anticipated to replace traditional graft procedures for treatment of tissue and organ defects. In tissue engineering, temporary implants are used to regenerate new tissue. A porous polymer matrix is often used as a guide for the cells to adhere, proliferate, and grow on (1). In order to obtain successful tissue generation *in vitro*, a scaffold with a highly specialized properties such as their topography, surface chemistry, mechanical properties, and degradation rates is required (2,3). These factors are critical and influence on the ability of cells to colonize a scaffold and eventually form an organized tissue construct. Implantation *in vivo* requires the scaffold to be biocompatible and to integrate within the surrounding natural tissue, and also to be completely eliminated from the host via biodegradation over a favorable time scale (4).

The demands on the scaffold materials are explicit for each specific application for which they are intended, giving rise to the need for a broad array of material properties. Protein-based materials such as silk protein, fibroin (fibrous protein), and sericin (degumming protein) have generated much interest in the biomedical and biotechnological fields due to their unique properties (5–8). Many researchers have successfully formed fibroin scaffolds for vascular tissue, connective tissue, and bone regeneration (9–11) whereas sericin scaffolds have been applied in skin substitution (12). Sericin is considered to be a waste material in textile manufacturing. However, its characteristics include high biocompatibility and biodegradability, low toxicity, and high hydrophilicity, and its low cost has also increased interest in the use of this compound in tissue engineering. Its potential and existing applications are extensive in medical, pharmaceutical, and cosmetic sectors. The effects of their production methods and the sterilization process used are often overlooked, even though they might have significant effects on the physical and biological properties of sericin scaffolds.

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A sterilization process is essential for every material or device for clinical use and the efficacy of sterilization techniques must be confirmed. Biomaterials with a complex architectures and hydrolytic degradation mechanisms from scaffolds may be easily damaged by harsh sterilization processes. Since sterilization treatments may adversely affect the material properties, any changes must be fully characterized and accounted for in the manufacturing process. These alterations may be detrimental or beneficial changes at the cellular level with respect to cell–surface interactions (13). Nevertheless, the challenge remains to discover an efficient and non-destructive sterilization protocol for biomaterial scaffolds which preserves their 3-D structure and ability to facilitate repair.

Biomedical devices prepared from biodegradable polymers are usually sterilized by ethylene oxide (EtO) because other sterilization procedures such as heat, steam or acid can cause extensive deformation of the devices and accelerate polymer degradation (14,15), whereas very little degradation occurs when EtO is used (16). However, in some polymers, EtO sterilization may lead to changes in the dimensions of scaffolds through shrinkage (16). Disinfection by ethanol is often used *in vitro* and is shown to produce no morphological or chemical damages to polyester scaffolds (16). Ethanol is considered as a strong immediate bactericidal activity (17) and virucidal activity at high concentration (ca. 95%) (18). It also has broad activity against most fungi-including yeasts and dermatophytes but virtually has no sporicidal activity (19). However, no study on ethanol as sterilize agent has been performed on protein-based biomaterials. Gamma irradiation is a common technique for sterilizing polymeric implants (20). Since scaffolds usually have a porous structure, a sterilization method is required that can penetrate such materials without leaving residues. Gamma irradiation is highly penetrative, although it causes a decrease in the tensile strength of hydrophobic polyurethanes (21). In some cases, the properties and performance can be negatively affected because of material degradation or induced cross-linking (22). Again, no study has been performed on the effect of gamma irradiation on protein-based biomaterial scaffolds.

Therefore, it is clear that each method will have advantages and limitations, the choice of a particular method must be carefully considered. The purpose of this study was to investigate changes in the physical properties of silk sericin scaffolds after sterilization by EtO, gamma irradiation, and ethanol treatment. Since the different sterilization processes may have different effects on various types of materials and different scaffold processing techniques, the release of sericin from scaffolds which may be beneficial for collagen production in wounds was also investigated.

MATERIALS AND METHODS

Preparation of a Three-Dimensional Silk Sericin and Polyvinyl Alcohol Scaffolds

The fresh, white-shell cocoons of *Bombyx mori* were kindly supplied by Chul Thai Silk Co., Ltd. (Petchaboon province, Thailand). Silkworm cocoons were produced in a controlled environment. After cutting the cocoons into pieces (about 5 mm^2), silk sericin was extracted using a high temperature and pressure degumming technique by mixing the silkworm cocoons with purified water (1 g of dry silk cocoon/30 mL of water) and the samples were autoclaved (SS-320, Tomy Seiko Co., Ltd., Tokyo, Japan) at 120°C for 60 min. After filtration through a membrane to remove fibroin, the sericin solution was concentrated until the desired concentration (approximately 7% *w*/*v* as measured by the BCA Protein Assay Reagent, Pierce, Rockford, IL, USA) was achieved.

Polyvinyl alcohol (PVA, Ajax Finechem, New South Wales, Australia, molecular weight 77,000–82,000) was dissolved at 80°C with constant stirring for about 4 h until it was completely dissolved to a concentration of 6% *w*/*v*. Glycerin, added as a plasticizer and to improve the scaffolds functional properties, was blended together with sericin and the PVA solution at room temperature for at least 30 min to make a final wet composition of 3% *w*/*v* sericin, 2% *w*/*v* PVA, and 1% *w*/*v* glycerin, which was then poured into a petri dish and frozen at −20°C, followed by lyophilization (Heto LL 3 000 lyophilizer, Allerod, Denmark) for 72 h.

EtO Sterilization and Gamma Irradiation

Scaffolds for EtO sterilization and gamma irradiation were packed in self-sealing sterilization pouches (saf-T-seal®, MD Industries, Maharashtra, India). Ethylene oxide sterilization was achieved in a 100% ethylene oxide atmosphere at 55°C for 3 h. The samples were then exposed to air for 12 h. Gamma irradiation sterilization was achieved at a dose of 25 kGray ^{60}Co at 55°C.

To validate ethylene oxide cycles used in the sterilization, overkill method has been performed using *Bacillus atrophaeus* (3M™ Attest™ 1294 rapid readout biological indicator for ethylene oxide, 3M Technologies Pte Ltd, Singapore). The overkill method is based on demonstrating that the sterilization of a microbial challenge (biological indicator) exceeds the challenge posed by the bioburden of the product. In our case, exposure of ethylene oxide for 3 h shows negative result of biological indicator which indicated that the ethylene oxide cycles are valid.

Ethanol Sterilization

The ethanol sterilization procedure was performed using a modified method from Karp *et al.* (23). A 200-mL 70% (*v*/*v*) ethanol solution (diluted from 96% *v*/*v* ethanol (Fisher Scientific UK Ltd, Loughborough, UK) with distilled water) was added to the scaffold. The samples were treated for 5 min and were subsequently rinsed three times with 100 mL deionized water.

Sterility Testing

All scaffolds were tested for sterility immediately following sterilization using a previously described procedure (24). Briefly, the samples were immersed in a Nutrient Agar Broth (Himedia Laboratories, Mumbai, India) to cultivate fastidious microorganisms and maintained under agitation at 25°C for 48 h. An untreated scaffold was used as the negative control while Allevyn (a commercial scaffold-like product, Smith & Nephew Medical Limited, London, UK) was used as the

positive control. Clouding of the broth after 48 h indicated contamination and inefficient sterilization, while a clear, uncontaminated broth indicated efficient sterilization, producing a sterile product. All experiments were performed in triplicate.

Bioburden Challenge Test

Test organisms that included Methicillin-resistant *Staphylococcus aureus* (MRSA; DMST 20645 lot no. 3273, DMST Culture Collection, The National Institute of Health, Department of Medical Sciences, Ministry of Public Health, Thailand) and *Bacillus subtilis* (ATCC 6633 DMST 15896 lot no. 3479, DMST Culture Collection, The National Institute of Health, Department of Medical Sciences, Ministry of Public Health, Thailand) were suspended in tryptic soy broth (TSB) to provide a final concentration of 10^2 and 10^4 colony forming unit (cfu)/mL. The inoculation of the test carrier was performed by using micropipette to place 20 μL of the test suspension on the surface of scaffolds and left to dry in the incubator for 18 h at 37°C (25). The scaffolds were then sterilized by ethanol, EtO, and gamma irradiation as previously mentioned protocol. After sterilization, the scaffolds were then transferred to test tubes containing TSB which were incubated at 25°C for 7 days. The turbidity of TSB was measured every day using UV spectrophotometer at wavelength 625 nm and using McFarland Standards as a reference to estimate the number of colonies. Unsterilized scaffolds with test suspension was used as positive control while sterilized scaffold without test suspension was identified as negative control, respectively. All experiments were performed in triplicate.

Changes in Morphology and dimensions

Scanning electron micrographs were taken on a JSM-5 800LV (JEOL JSM-5410LV, Tokyo, Japan) scanning electron microscope (SEM) at an acceleration voltage of 15 keV after cutting the scaffold into pieces, mounting onto aluminum stubs and sputter coating with gold at a 10–20 nm thickness. Sterilized and degraded scaffolds were checked for changes in gross (i.e., outer) scaffold dimensions (n=3 for each group), and the percentage of volume changes was calculated. The outer dimensions of the scaffolds were measured using a Micrometers Series 169-Non-Rotating Spindle Type (Mitutoyo Corporations, Kawasaki, Japan). All experiments were performed in triplicate.

Pore Size Measurement of Scaffolds

Pore size was measured by following a modified method from Kang *et al.* (26). The major and minor diameters of each scaffold were measured using a stereomicroscope equipped with an optical micrometer. The pore size was calculated as the geometric mean of the major and minor diameters, respectively. At least 100 pores were assessed, and the values presented indicate the mean ± standard deviation.

Fourier Transforms Infrared Spectroscopy

The FT-IR spectra of the compounds were recorded on a Perkin Elmer spectrometer by the KBr pellet technique. The sericin patch was ground into small pieces in mortar. For a verification of the compound structure, a KBr pellet was prepared by grinding of about 2 mg sericin samples with 200 mg KBr. The peak areas were calculated by the baseline technique. The standardization procedure was based on the preparation of a calibration curve between the integrated areas and the concentration of the salt in the KBr pellet. Infrared spectrum was recorded between 4,000 and 400 cm^{-1}.

Circular Dichroism

Circular dichroism (CD) was performed on a JASCO J-715 spectropolarimeter (Jasco Inc., Tokyo, Japan) using 0.1 cm path length quartz cell at temperature 20°C. The sample was analyzed in DDW. The UV spectra were measured at a protein concentration of 1 mg/mL from 250 to 190 nm with speed scan at 50 nm/min, response time constant was 1 s, spectral bandwidth was 2.0 nm, step resolution was 0.5 nm, and sensitivity was 10 mdeg. Triplicate scans of the CD spectra were averaged to reduce error and noise, and the baseline correction was subtracted by DDW background spectra. The percentage of α-helix, β-sheet, turns, and random coil were determined using analysis function built into the Jasco-715 spectropolarimeter software.

Swelling Properties

Swelling studies were carried out according to Mandal *et al.* (27) with slight modifications. Briefly, the lyophilized scaffolds were accurately weighed in the dry state and then immersed in 10 mL of purified water. At 24 h, the scaffolds were carefully removed, and the amount of water contained in the scaffolds was precisely determined by weighing them in the swollen state. The experiments were performed in triplicate under the same conditions. The percentage of swelling of the scaffolds at equilibrium was calculated using the following equation:

$$\% \text{ swelling} = [(W_t - W_0)/W_0] \times 100$$

where W_0 is the weight of the dried test sample and W_t is the weight of the swollen test sample.

Degradation Study

Newly processed scaffolds were prepared with a 3-D rectangular geometry, allowing for an accurate measure of their initial mass. The scaffolds were then divided into four groups and each group treated either by EtO, gamma irradiation, or ethanol, respectively, while a non-sterilized scaffold was used as the control. Each treated sample was placed in a perforated Eppendorf tube and immersed in phosphate buffer solution (pH 7.4) at 37°C for 24 h. The ratio of the sample mass to buffer volume was 1:1,000 (w/v), and the pH of the buffer was routinely checked. After 24 h, all samples were removed from the buffer, washed repeatedly

with deionized water, and dried under a vacuum (P= 0.01 mmHg, 24 h at room temperature). The dried samples were evaluated for changes in mass by accurately weighing them and the percentage weight loss was calculated using the following equation:

$$\% \text{ weight lost after 24 h immersion} = [(W_0 - W_t)/W_0] \times 100$$

where W_0 is the weight of the dried scaffold at the beginning (before immersion) and W_t is the weight of the dried sample after 24 h immersion. All experiments were performed in triplicate.

Mechanical Properties of the Scaffolds

Tensile strength tests using the Universal Testing Machine (Hounsfield H10KM, London, UK) were performed on dumb-bell-shaped samples of 25 mm length and 6 mm width at a cross-head rate of 5 mm/min (ASTM D412-06a). The maximum stress and Young's modulus were obtained from the stress and strain tensile curve. All experiments were performed in triplicate.

The Release of Sericin from Sericin Scaffolds

The release profile of sericin from the scaffolds was plotted after placing the scaffold samples (diameter 35 mm) into PBS (pH 7.4) at room temperature with continuous stirring in a closed-container. Samples (1.5 mL) were removed at different time points 0, 1, 15, 30 min, 1, 2, and 3 days, and the amount of sericin was measured immediately after sampling using a BCA protein assay kit (Pierce, Rockford, IL, USA). Briefly, the leached protein samples were collected, mixed with BCA reagents, and vortexed. The absorbance was measured at 562 nm and the amount of protein released was compared with a bovine serum albumin standard curve. All experiments were performed in triplicate.

Statistical Analysis

Data was expressed as the mean ± SD. The statistical significance was determined by paired and unpaired Student's t tests together with ANOVA. A value of $p<0.05$ was considered to be significant.

RESULTS

Effectiveness of Sterilization Methods and Scaffold Structure

After 48 h incubation, the untreated controls all produced signs of growth during sterility test whereas the Allevyn remained free of growth throughout the same time period. However, the broth in the tubes containing the ethanol-treated scaffold showed opacity after 24 h while the gamma-irradiated and EtO-treated scaffolds showed successful sterilization throughout the treatment durations. These results indicate the

Table I. Amounts of Microorganism Growth on Sericin Scaffolds

	Day						
	1	2	3	4	5	6	7
Positive control							
MRSA 10^2 cfu/mL	−	−	−	+	++++	++++	++++
MRSA 10^4 cfu/mL	−	−	++	++++	++++	++++	++++
B. subtilis 10^2 cfu/mL	−	++	++++	++++	++++	++++	++++
B. subtilis 10^4 cfu/mL	−	+++	++++	++++	++++	++++	++++
Negative control							
EtO	−	−	−	−	−	−	−
Gamma irradiation	−	−	−	−	−	−	−
EtOH	−	++++	++++	++++	++++	++++	++++
MRSA 10^2 cfu/mL							
EtO	−	−	−	−	−	−	−
Gamma irradiation	−	−	−	−	−	−	−
EtOH	−	−	−	−	−	−	−
MRSA 10^4 cfu/mL							
EtO	−	−	−	−	−	−	−
Gamma irradiation	−	−	−	−	−	−	−
EtOH	−	−	−	−	+	++++	++++
B. subtilis 10^2 cfu/mL							
EtO	−	−	−	−	−	−	−
Gamma irradiation	−	−	−	−	−	−	−
EtOH	−	−	+	++++	++++	++++	++++
B. subtilis 10^4 cfu/mL							
EtO	−	−	−	+	++++	++++	++++
Gamma irradiation	−	−	−	−	−	−	−
EtOH	−	+++	++++	++++	++++	++++	++++

− no growth, + amount of microorganism below 1.5×10^8 cfu/mL, ++ amount of microorganism between 1.5 and 6×10^8 cfu/mL, +++ amount of microorganism between 6 and 12×10^8 cfu/mL, ++++ amount of microorganism more than 12×10^8 cfu/mL

suitability of the sterilization techniques tested to these particular scaffolds. Table I shows the result of bioburden test which indicated that ethanol treatment is the least effective in sterilizing scaffold, similar to the result during sterility test, while gamma irradiation is the most effective method as shown by clear broth media after challenging with high bacterial suspension even after 7 days incubation.

Figure 1 shows the SEM cross-section images of scaffolds both before and after the sterilization treatment. Figure 1a is an image of the untreated control sample, which shows homogeneity throughout the sample, while Fig. 1b and c represent EtO- and gamma-irradiated scaffolds, respectively. The pore size in both the EtO- and gamma-irradiated scaffolds appears more irregular compared with the untreated sample and were more fragile under SEM; magnifications above ×2,500 resulted in damage to the samples within seconds. Ethanol treatment had a large impact on the scaffolds when compared to the controls. The samples sterilized in ethanol shows reduced porosity and increased of surface wrinkles (Fig. 1d).

Comparing Sterilization Techniques for Changes in Dimension and Pore Size

In all cases, the structure of the scaffolds changed during treatment. The samples sterilized by EtO, gamma irradiation, and ethanol were compared to untreated samples in regard to changes in dimension, shown as volume and pore size changes, as summarized in Fig. 2 and Table II, respectively. Ethylene oxide and gamma irradiation slightly altered the dimensions of the sericin scaffold whereas ethanol-treated samples shrank significantly compared to the other samples. After ethanol sterilization, there was a loss of approximately 80% of their initial volume. The ethanol-sterilized samples also looked softer than all of the other sterilized samples. Table II provides details of the pore size of sericin scaffolds both before and after sterilization treatment, indicating that ethanol-treated samples have a significantly smaller pore size compared with the control and EtO- and gamma-irradiated samples. Ethylene oxide and gamma irradiation slightly reduced the pore size but not to a significant level.

FT-IR Results

The FT-IR spectra of the sericin samples are shown in Fig. 3. These compounds exhibited the amide absorption bands of protein at ca. 3,286, 2,941, 1,664, 1,535, 1,415, and 1,046 cm^{-1}. The peak at 3,286 cm^{-1} corresponds to N–H stretching vibrations, and this may be caused by amide structure (only one stretching of secondary amine). The amide absorption primarily attributes to the C=O group peaks which can be seen

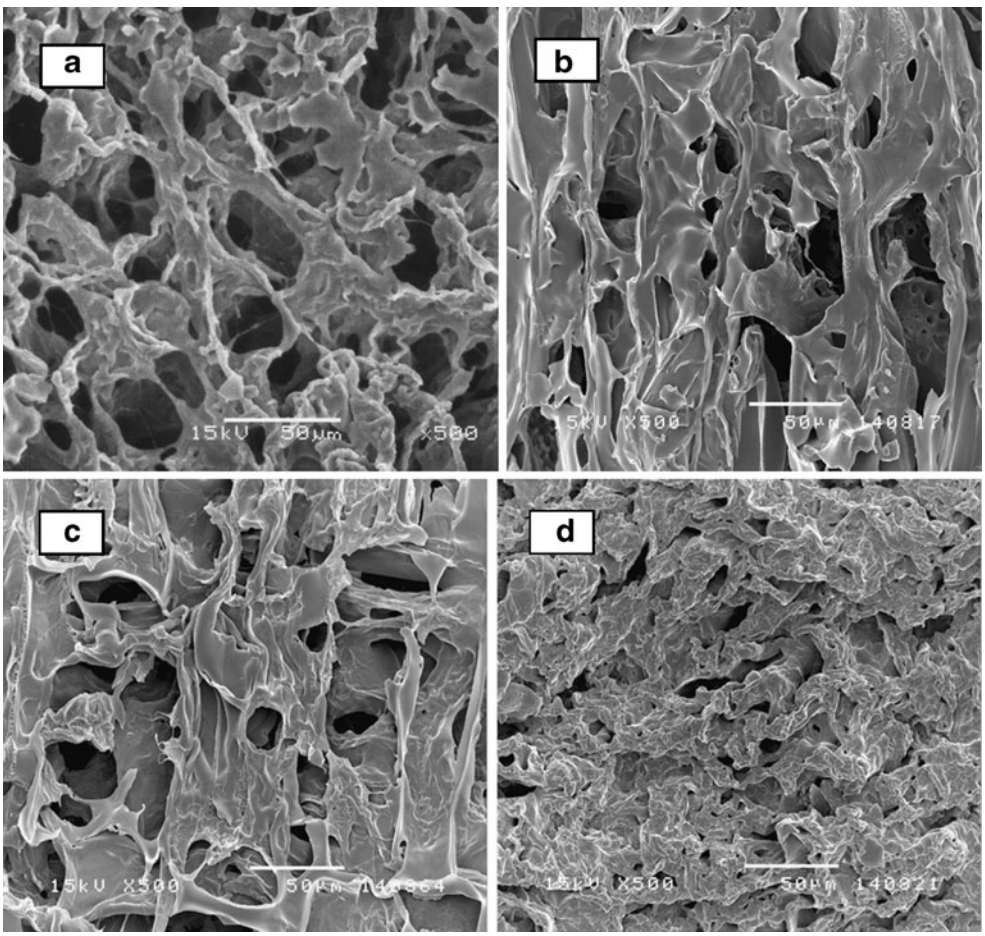

Fig. 1. SEM cross-section images of scaffolds. **a** sericin scaffold (unsterile), **b** ethylene oxide-treated sericin scaffold, **c** gamma irradiation-treated sericin scaffold, **d** ethanol-treated sericin scaffold

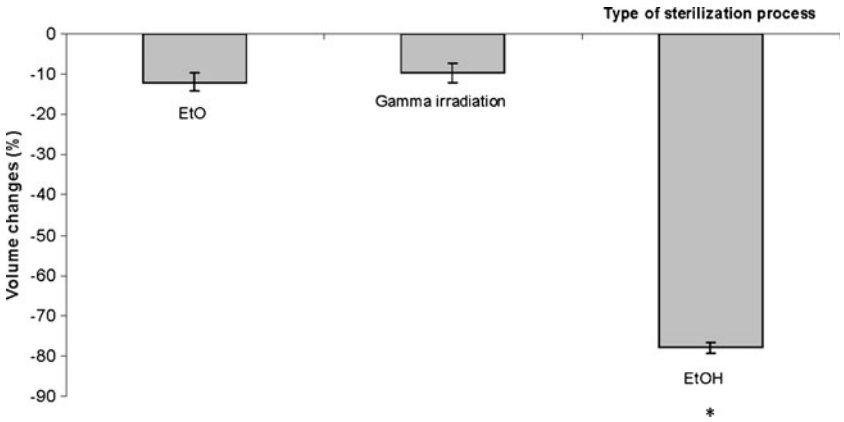

Fig. 2. Changes in the dimensions (shown as percentage of volume changes) of sericin scaffolds before and after sterilization treatment. *$p<0.05$ indicates significant differences

at 1,664 cm^{-1}. N–H bending and C–N groups exhibit characteristic band at 1,535 cm^{-1}. The band at 1,415 cm^{-1} is attributed to C–H and O–H bending, and the band between 1,099 and 1,046 cm^{-1} reveals to be C–OH vibrations stretching. The spectra of each sample are very similar to one another. It is postulated that the chemical structure of sericin scaffold did not change dramatically during treatments with ethanol, EtO, or gamma irradiation.

CD Result

The far-UV CD spectra of sericin scaffolds are shown in Fig. 4. They revealed strong negative peaks at 196 nm of untreated (3S2P1G) and ethanol-treated scaffolds suggesting a random coiled structure. However, in EtO-treated and gamma-irradiated scaffolds, the negative peaks shifted to 195 and 192 nm, respectively. A negative band at 220 nm suggested the presence of β-sheets in all samples. Absence of any positive bands around 190 nm excluded the presence of any α-helix in any sericin scaffolds in this study. The percentages of different secondary structures in the sericin scaffolds indicated 36–38% of β-sheet, 12–18% β-turn, and 45–49% random coil in untreated and ethanol-treated scaffolds. Whereas in EtO-treated and gamma-irradiated scaffolds, the percentage of β-sheet decreased to 28–29%, the percentage of random coil slightly decreased and percentage of β-turn slightly increased. Overall, the structures of sericin in this study were random coil, β-sheet, and β-turn. The treated samples did not change their conformation from the untreated scaffolds.

Table II. Pore Size of Sericin Scaffolds Before and After Sterilization Treatment

Sample	Pore size (μm)
Untreated (control)	43.81±18.62
Ethylene oxide	40.75±15.15
Gamma irradiation	40.30±13.22
Ethanol treatment	24.29±11.88*

*$p<0.05$, significant differences compared with other scaffolds

Swelling Properties

The swelling property of the sericin scaffolds before and after the sterilization treatment is shown in Fig. 5. All sericin scaffolds can absorb moisture efficiently, as shown by the high water holding capacity, including the untreated scaffold. However, the untreated scaffold became too fragile to be handled and broke into pieces while the ethanol-treated scaffold exhibited the highest intact structure after immersion for 24 h. The swelling property of the ethanol-treated scaffold was similar to that of the control, whereas gamma irradiation significantly increased the swelling property of the sericin scaffold. There was an approximately twofold swelling of the gamma-irradiated sample compared to the control after 24 h immersion. Ethylene oxide also increased the swelling property of the sericin scaffold compared to the untreated sample, but not to a significant level.

Degradation Study

Figure 6 represents the percentage of weight loss of the sericin scaffolds after 24 h immersion in water. The control lost approximately 90% of its weight after immersion which was significantly difference compared to the other samples, and as mentioned above, the control samples also had the least intact structure. All sterilization treatments reduced the loss of the scaffolds structure during immersion for 24 h, especially the ethanol-treated scaffold, which showed the smallest weight loss after immersion. The scaffolds treated with EtO and gamma irradiation lost approximately 60% and 70% of their initial weights, respectively, while the ethanol-treated scaffold only lost 40% compared to its initial weight.

Mechanical Properties of the Scaffolds

Figure 7 shows the mean maximum stress resistance of the sericin scaffolds before and after the sterilization treatments. The untreated scaffold exhibited the highest maximum stress resistance and was similar to the maximum stress resistance produced by the EtO-treated scaffold. Treatment by gamma irradiation and ethanol significantly reduced the maximum stress resistance of the sericin scaffolds. The

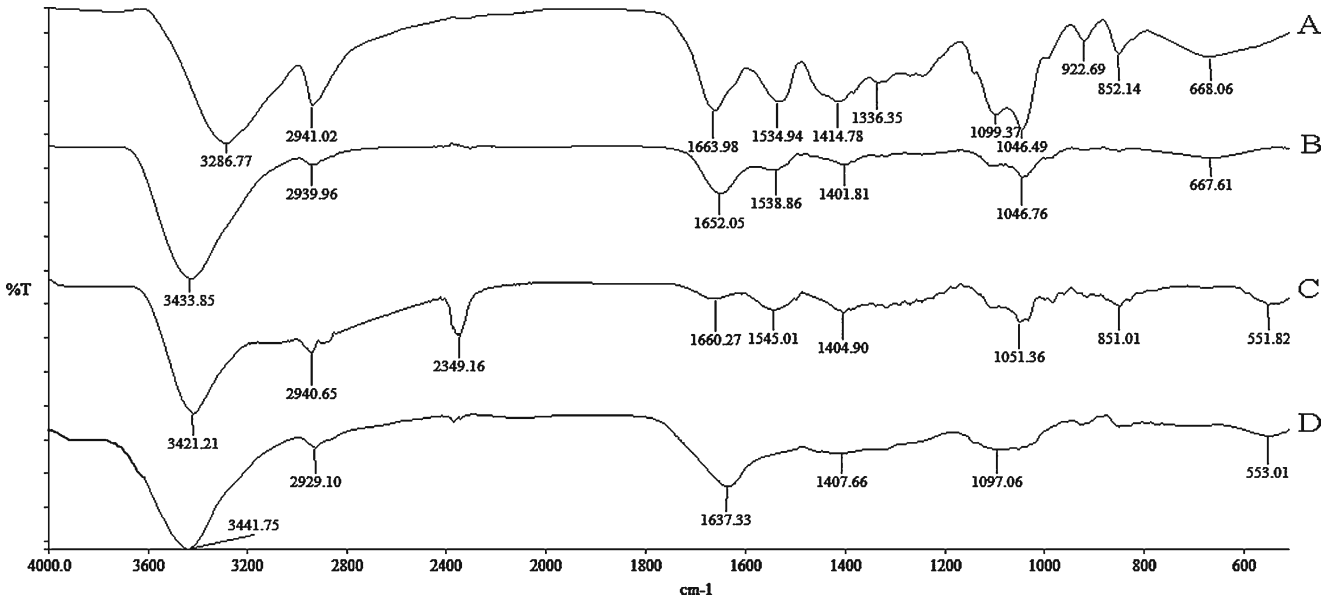

Fig. 3. FT-IR spectra of sericin scaffold before sterilization (*A*) and after sterilization with 70% ethanol (dry; *B*), ethylene oxide (*C*), and gamma radiation (*D*)

maximum stress resistance of the ethanol-treated scaffold was significantly lower when compared to the untreated and EtO-treated samples, which indicated that the untreated and EtO-treated scaffolds possessed relatively higher tensile strengths when compared to the gamma irradiation and ethanol-treated scaffolds.

Young's modulus of the sericin scaffolds before and after sterilization treatment are shown in Fig. 8. Gamma irradiation, similar to the ethanol-treated sericin scaffolds, had a lower Young's modulus compared with the untreated and EtO-treated scaffolds. The Young's modulus of the untreated and EtO-treated samples showed significant differences compared to the gamma-irradiated samples. These data indicated that the control and EtO-treated scaffolds were stiffer, whereas the gamma-irradiated and ethanol-treated scaffolds had more flexibility.

The Release of Sericin from the Sericin Scaffolds

Figure 9 shows the amount of sericin released from the scaffolds before and after sterilization. The gamma-irradiated scaffold released the highest amount of sericin, whereas the ethanol-treated scaffold released the lowest amount of protein, which was significantly different compared to the other scaffolds. Moreover, the amount of sericin released from the gamma-irradiated scaffold was significantly different from the amount of sericin released from the EtO-treated scaffold. The maximum amount of protein leaching from all

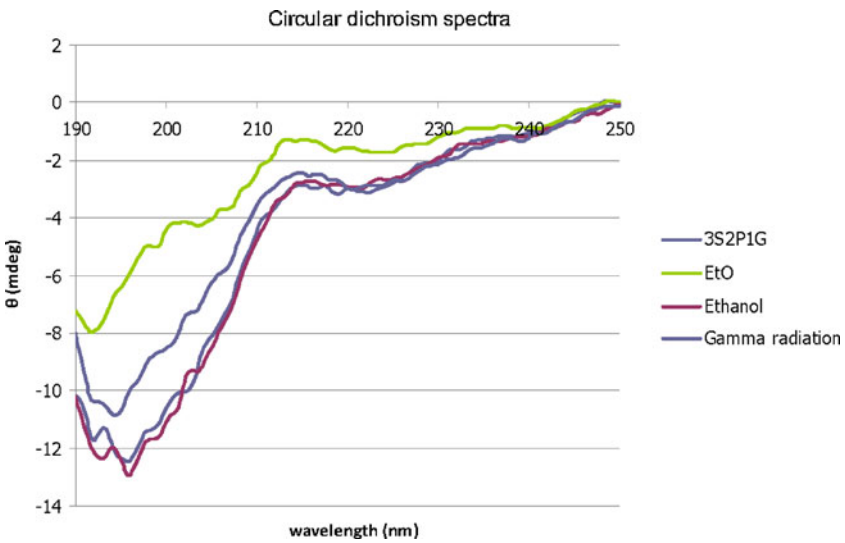

Fig. 4. The far-UV circular dichroism spectra of sericin scaffolds

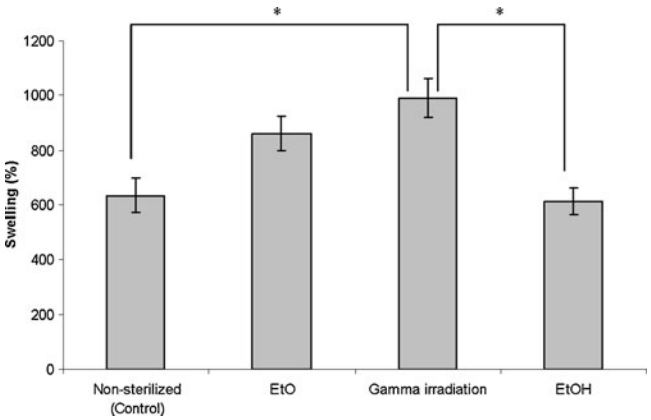

Fig. 5. The swelling property of sericin scaffolds before and after sterilization treatment. *$p<0.05$ significant differences

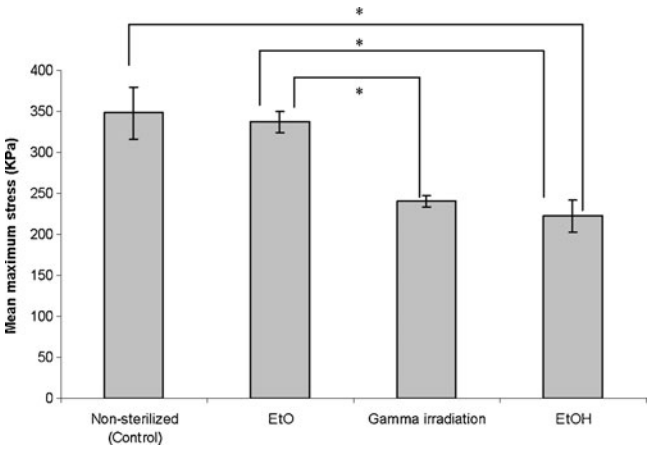

Fig. 7. The mean maximum stress resistance of the sericin scaffolds before and after sterilization treatment. *$p<0.05$ significant differences

the scaffolds was observed within 12 h. The fractions of protein released from the untreated, gamma-irradiated and EtO-treated scaffolds were approximately 3.15%, 3.44%, and 2.69%, respectively, while only 1.58% of sericin was released from the ethanol-treated scaffold.

DISCUSSION

Sericin, a silk protein, can be successfully formed 3-D scaffolds after mixing with PVA and glycerin, which can be used in tissue engineering. However, all biomaterials have to be efficiently sterilized before use. The sterilization method must be carefully selected since it can induce undesirable changes to the characteristics of the biomaterial that may affect cell–material interactions. Since sericin is mainly protein, heat, and radiation can alter its tertiary structure, which may result in its characteristics being changed. To the best of our knowledge, the published literature contains no reports on the sterilization of protein-based scaffolds.

Our results indicated the significant effects of the sterilization process on sericin scaffolds. Sterilization effectiveness was determined qualitatively by the absence of signs of growth after a 48-h incubation period. Growth was indicated by a change in opacity of the culture medium. Ethanol treatment showed the least effective sterilization method for the sericin scaffold. This result may have been due to the fact that ethanol is not actually a sterilizing agent, although it is a good disinfectant (due to its dehydration action and protein coagulation effect, which destroys membranes and denatures proteins); this solvent does not act on the endospores of many bacteria (28). Because of this, ethanol should only have a limited use as a surface-sterilizing agent. EtO and gamma irradiation indicated a successful sterilization was achieved. However, gamma irradiation is superior to EtO during challenge test.

Dimensional changes, expressed as percentage changes in volume, were observed for all samples. Gamma irradiation and EtO treatment decreased their initial volume by approximately 10%, whereas the ethanol-treated scaffolds decreased by approximately 80% of their initial volume. The effect of ethanol and EtO treatment on the dimension of the scaffolds are similar to results reported by others (16). While the overall dimensional change of all of the sterilized samples decreased, their degradation profiles were distinct: as shown in the results, the ethanol-treated scaffolds shrank considerably during the sterilization process, but remained at about 60% of their initial volume for the entire duration of the degradation study. The gamma-irradiated samples did not change their dimensions significantly during the sterilization

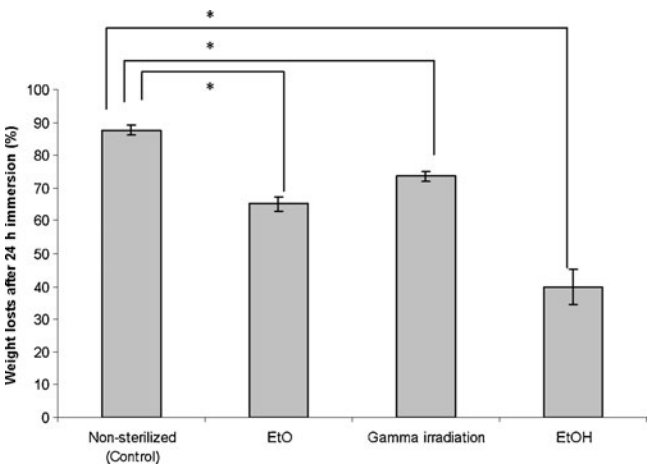

Fig. 6. Percentage of weight loss of sericin scaffolds before and after sterilization treatment, after 24 h immersion. *$p<0.05$ significant differences

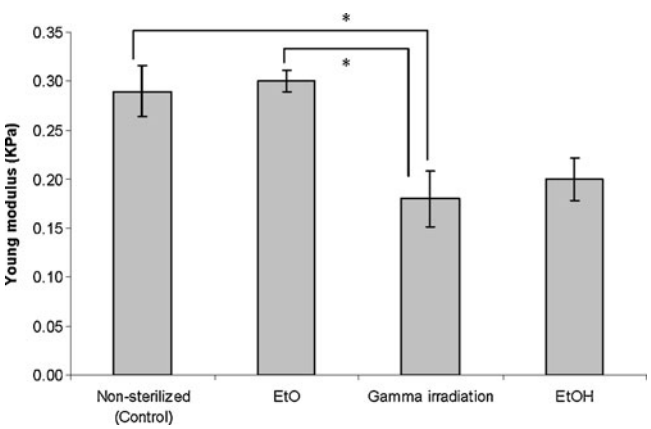

Fig. 8. Young's modulus of the sericin scaffolds before and after sterilization treatment. *$p<0.05$ indicates significant differences

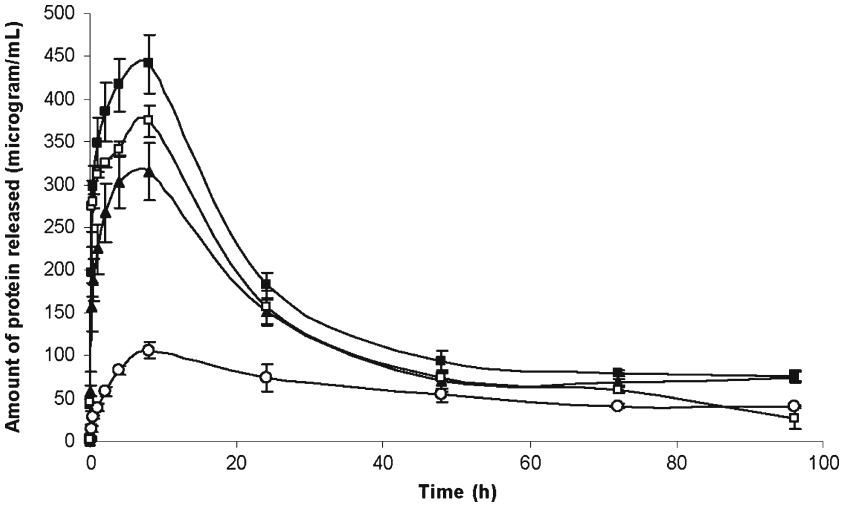

Fig. 9. The amount of sericin released from the scaffolds before and after sterilization: *unfilled squares* represents the untreated scaffold (control), *filled triangles* represents the EtO-treated scaffold, *filled squares* represents the gamma-irradiated scaffold, and *unfilled circles* represents the ethanol-treated scaffold

process, but they only remained at about 30% of their initial volume during the degradation study. Even though EtO and gamma irradiation were effective in the sterilization of the sericin scaffolds, the gamma-irradiated scaffolds are suitable only in low moisture environments in order to avoid its high degradation in the presence of water.

After being sterilized with EtO, gamma irradiation, and ethanol, the SEM images revealed that the sericin scaffolds responded to EtO and gamma irradiation in a similar manner, whereas the ethanol treatment produced significant effects on the scaffold. However, the physical appearance of the ethanol-treated scaffold did not show any imperfections, only a few surface wrinkles. After exposure to gamma irradiation and EtO, no significant differences were observed from before sterilization. The SEM and pore size measurements indicate that the ethanol treatment significantly reduced the pore size of the sericin scaffolds. However, the pore sizes of all of the sericin scaffolds in this study still remained within the range of 20–50 μm, which is suitable for the proliferation of keratinocytes (29).

The swelling property of a scaffold is essential for effective wound healing since it confers high exudates absorption capacity. However, the scaffolds should be stable and intact after moisture absorption. The reduced percentage weight loss of all of the treated samples indicated that the EtO, gamma irradiation, and ethanol also have an effect on the cross-linking or structure of sericin and results in more intact structures compared to the untreated samples. Even though most of the scaffolds showed a considerable weight loss after immersion for 24 h, in clinical applications, the moisture contained in wounds will not be a significant amount and the sericin scaffold should remain intact.

The mechanical properties of the scaffolds were influenced by the tested sterilization methods. Tensile strength was, in all cases, reduced after sterilization. The maximum stress resistance of all samples showed statistically significant losses when compared to the untreated scaffolds. The mechanical properties of the gamma-irradiated and ethanol-treated samples were quite similar: both scaffolds should be more flexible than the EtO-treated and untreated samples, but at the same time, they might break easily after receiving a certain amount of stretch. The results found here differ from those reported by others, which indicated that gamma irradiation reduced chitin-glycerol membrane elasticity significantly (28). The data of the present study confirmed that the effect of sterilization methods on physical properties, especially mechanical strength, may be different in various materials.

From our results, the sericin scaffold is highly elastic and flexible, which should be useful in wound care. Nevertheless, the low Young's modulus of the sericin scaffold may limit its use in tissue engineering: this similar mechanical property was found in other scaffolds such as the fibroin/hyaluronic acid composite and the chitosan scaffold, which can be used for neural tissue engineering and dermal substitution (30,31), but they are not suitable for bone tissue engineering.

Sericin can promote wound healing via activating collagen production: as the low levels of sericin released from the scaffold can be beneficial but at the same time, the matrix should also be stable. In all cases, protein release from scaffolds occurred simultaneously with mass loss and all of the scaffolds released sericin in a time-dependent manner. The release of PVA also occurred simultaneously with sericin as shown in previous study (12). The gamma-irradiated scaffold released the highest amount of sericin, which was even higher than that released from the untreated sample. This may have been due to a decrease in the molecular weight of the sericin after exposure to gamma irradiation, which is similar to results found in bovine serum albumin (32). The hydrolysate or smaller molecular weight of sericin can also support cellular viability especially in the cellular-damaged condition (33) and still promote collagen production as shown in heat-degraded sericin (34). The decrease in molecular weight, or less aggregation, allows protein to become released easily, which is another advantage of the gamma irradiation method for the sterilization of sericin scaffolds.

In contrast, ethanol released the smallest amount of sericin from the scaffold and exhibited a more intact

structure. The shrinkage and smaller pore size in the ethanol-treated scaffold indicated that there is a high entrapment of sericin itself and, with other components such as PVA in the scaffold, these result in less sericin being available for release.

Knowledge of the effects of sterilization allows the incorporation of these effects into the scaffold design. Some sterilization methods may show advantages or disadvantages compared to others. However, due to the delicate nature of sericin and the required degradability of tissue-engineered scaffolds, all factors related to their physical and biological properties after the sterilization process need to be considered.

CONCLUSIONS

Our results have indicated that sterilization processes have significant effects on the sericin scaffolds. Both gamma irradiation and EtO successfully reduced the chances of the sample becoming infected, but gamma irradiation is superior to EtO since it can sterilize scaffold for longer period of time especially in highly contaminated condition, whereas the ethanol treatment was not suitable for sterilizing this protein-based scaffold. Gamma irradiation and EtO did not cause any difference in the pore size or dimensions of the scaffold, whereas ethanol significantly reduced both pore size and the dimensions, as shown by the volume changes. With regard to the swelling property, the gamma-irradiated scaffold had the highest ability to absorb moisture while the ethanol-treated scaffold absorbed the least, but it remained the most intact scaffold after immersion in water for 24 h. The mechanical properties of gamma-irradiated and ethanol-treated scaffolds seem to be appropriate for further applications since they showed more flexibility. The highest amount of sericin released from a scaffold was found in the gamma-irradiated samples: this will be beneficial for activating collagen formation in wounds. While the results showed that none of the sterilization methods are ideal in that all methods caused some changes to the structural and physical properties, gamma irradiation should be the most appropriate procedure for sterilizing the sericin scaffolds for use in the tissue-engineering field.

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