JSTR
ASEAN Journal of Scientific and Technological Reports

Research article

Fabrication, Characterization, and Biocompatibility Study of Gelatin-Blended Fibroin Scaffold

Kamonchanok Naksuwan[1], Sukanya Chaipayang[2], Pollawat Charoeythornkhajhornchai[3], and Santi Phosri[4*]

[1] Department of Chemical Engineering, Program in Bioengineering, Faculty of Engineering, Burapha University, Chonburi, 20131, Thailand; Mewkamol@gmail.com
[2] Synchrotron Light Research Institute, Nakhon Ratchasima, 30000, Thailand; Sukanya@slri.or.th
[3] Department of Advanced Materials Engineering, Faculty of Engineering, Burapha University, Chonburi, 20131, Thailand; Pollawat.ch@eng.buu.ac.th
[4] Department of Chemical Engineering, Faculty of Engineering, Burapha University, Chonburi, 20131, Thailand; Santi.ph@eng.buu.ac.th
* Correspondence: Santi.ph@eng.buu.ac.th

Citation:
Naksuwan, K.; Chaipayang, S.; Charoeythornkhaijhornchai, P. ; Phosri, S. Fabrication, characterization, and biocompatibility study of gelatin-blended fibroin. *ASEAN J. Sci. Tech. Report.* **2023**, *26*(2), 67-76. https://doi.org/10.55164/ajstr.v26i2.248655.

Article history:
Received: February 27, 2023
Revised: March 28, 2023
Accepted: March 29, 2023
Available online: March 31, 2023

Abstract: This research investigates the biocompatibility of gelatin-blended fibroin scaffold fabricated by freeze-dry process. The morphology showed interconnected spongy spheres and polygons shapes with a pore size of 14.144 ± 11.31 and 19.4822 ± 18.71 micrometers, respectively. The formula ratio of fibroin:gelatin of 8:2 contained more random coil, while the formula ratio of fibroin:gelatin of 7:3 exhibited more β-sheet structure. The result revealed that gelatin influences fibroin conformational transition from random coil to beta, which might result from better hydrophobic characteristics. The surface of the formula ratio of fibroin:gelatin at 8:2 and 7:3 was 125.88 ± 9.85 and 130.07 ± 3.72 degrees, indicating the formula ratio of fibroin:gelatin at 7:3 had more hydrophobicity than the formula ratio of fibroin:gelatin at 8:2. The biocompatibility of scaffolds was determined for both formulas of human keratinocyte (HaCaT) and African green monkey kidney (Vero) cell lines by MTT assay. The formula ratio of fibroin: gelatin at 8:2 with various concentrations of 62.50, 125, 250, 500, and 1,000 μg/mL had cell viability percentages of 96.31, 101.74, 97.81, 104.15, and 103.22%, respectively. The formula ratio of fibroin:gelatin at 7:3 with various concentrations of 62.50, 125, 250, 500, and 1,000 μg/mL had cell viability percentages of 106.38, 104.28, 108.54, 97.42, and 97.65%, respectively. The growth of cell lines showed better performance with the hydrophilic characteristics of the scaffold, and it did not make it toxic to the HaCaT and Vero cell lines. These results demonstrated the possibility of gelatin-blended scaffold as a supporting material for skin tissue engineering, especially for wound healing.

Keywords: Biocompatibility; scaffold; fibroin; gelatin

1. Introduction

Diseases and accidents lead to injuries and damage to tissues and organs, especially skin tissue. The treatment of skin tissue generally focuses on tissue and organ transplantation [1]. Transplantation is the standard method of treating extensive injuries or wounds to the injured part of the skin tissue. However, tissue grafts and organ transplantation methods may not achieve this entirely because they do not have enough space for skin tissue. Therefore, tissue engineering has been developed to fabricate cell scaffolds for treating or closing acute and chronic wounds.

A scaffold is three-dimensional for cell culture that creates an artificial environment as the condition of cell growth in the body. It mimics the state of tissue physiology resulting in creation of new usable tissues. The scaffold could be made from natural or bio-based materials, an extensive study in tissue engineering applications. The scaffold production would be made the extracellular matrix of tissues that could maintain structural integrity in the body and eventually degradation. The scaffold product might be natural or synthetic materials with biodegradable and good biocompatibility, which does not cause any adverse effects on cell culture and does not stimulate the immune system or react against any organs of the human body [2]. Many materials are required for this application depending on biological, chemical, and mechanical properties.

The silk fibroin from the cocoon is a protein from natural fibers similar to the human body protein. It is a promising biomaterial for tissue engineering because it is biocompatible, degrades slowly, is chemically modified, and can be processed into various structures [3]. Likewise, it has excellent physical properties, being lightweight, strong, highly elastic, and thermally stable [3]. It can reduce inflammation and heal skin tissue wounds owing to good biocompatibility with human tissues [3-4]. Nevertheless, silk fibroin has poor cell adhesion, and some biomaterials that encourage cell adhesion, especially gelatin, to improve its properties. Gelatin is a protein product extracted from collagen by partial hydrolysis of collagen from animal skin, bone, and tissue. Arginine-glycine-aspartate (RGD) tripeptide in gelatin's bioactivities indicates cell-matrix interactions recognition binding site [5]. It could be the adhesive material between fibroin and skin tissue, promote cell growth [6], and have anti-inflammatory properties.

The silk-based scaffolds are a wide range. For example, freeze-dried sponges, nanofibers, and hydrogels have been investigated for tissue engineering and repair [7]. Therefore, this study aims to fabricate a gelatin-blended fibroin scaffold and confirm the physical properties and cell compatibility of blended scaffolds.

2. Materials and Methods

2.1 Silk fibroin extraction

Bombyx mori silk cocoons were purchased from Yasothon province. Silk fibroin (SF) was prepared by chopping 10 g of *Bombyx mori* silk cocoons into small pieces and degumming in 0.2 M of Na_2CO_3 twice the time at 100°C for 30 min. Next, the degummed cocoons were rinsed throughout to remove the glue-like sericin proteins with water. After degumming, silk fibers were dried in a hot air oven at 60°C overnight. The degummed silk fibers were then dissolved in a $CaCl_2/C_2H_5OH/H_2O$ solution (molar ratio 8:2:1) and continuously stirred at 70°C for 5 h. The obtained solution was centrifuged at 6,000 rpm to remove any impurities. The solution was then dialyzed with deionized water for three days to remove the salt that could remain in the solution. Finally, the SF solution was stored at 4°C until usage.

2.2 Fabrication of the composite scaffolds

Five percent of the gelatin solution was mixed with 5% of the silk fibroin solution at different ratios between fibroin and gelatin. Two formula scaffolds of fibroin:gelatin at 8:2 (v/v) and 7:3 (v/v), were investigated. Then, 2.5% of glutaraldehyde was added to the scaffold solution as a crosslinking agent. The answer was transferred to the 15 mL centrifuge tube and frozen at -40°C overnight, followed by freeze-dry process at -80°C for 48 h. The blended scaffolds were stored in aluminum foil at 4°C for characterization and testing.

2.3 Characterization

The microstructure of the blended scaffold was observed by scanning electron microscopy (SEM, ZEISS, LEO 1450 VP, USA). The standard procedures prepared samples: fixation in glutaraldehyde 2.5%vol at 4°C for 6 h and coating with gold by sputtering. The accelerating voltage of SEM was 10 kV. Fourier Transform Infrared Spectroscopy (FTIR) was used to characterize the intermolecular interactions between the components and conformational changes of each scaffold formula. The spectra of the individual and SF/Gelatin scaffolds were performed by a Bruker tensor 27 DTGS detector. Spectral data were measured using a platinum diamond ATR with the reflection mode of 64 scans at a resolution of 4 cm^{-1} over a measurement

range of 4,000-600 cm^{-1}. Data acquisition and analysis were performed by OPUS 7.5 software (Bruker Optics Ltd., Ettlingen, Germany).

The Image J software quantified porosity measurement with the analyze particles function. Axial and perpendicular sections of morphological change after preparation were analyzed. Furthermore, the hydrophilicity of the scaffolds was measured by the contact angle method. Five microliters of distilled water were dropped on the surface of each formula, and the contact angle was recorded after 2 s. The drop shape analysis software was used to calculate the contact angle. The results are shown as an average mean value with standard deviation.

The crystal structure of blended scaffolds was analyzed using wide-angle synchrotron X-ray scattering using 1.3 W of the beamline: SAXS/WAXS of the Synchrotron Light Research Institute (SLRI), Nakhon Ratchasima, Thailand that has energy 1.2 GeV. The distance between the sample and detector was 153 mm with LX170 as a detector. The Wide Angle X-ray Scattering (WAXS) intensity was obtained at a photon flux of 2×10^9 photons/s at 9 keV. The WAXS patterns were corrected using air as a background. The 2D WAXS patterns were reduced and radially averaged by SLRI (SAXSIT) staff-developed software to obtain 1D WAXS curves. The percentage of crystallinity (Pc) was calculated based on the data obtained from the WAXS profiles using the following equation (1)

$$\text{Percentage of crystallinity (Pc)} = (A_c/A_c+A_a) \times 100 \quad (1)$$

Where A_c is, and A_a are the areas under the crystalline peak of interest and the amorphous halo, respectively [8].

For the biocompatibility assay, the scaffold was placed into 1.5 mL micro centrifugal tubes and sterilized under UV light for 15 min. After that, FBS-free DMEM was added from a stock solution at 20 mg/mL for 24 h to extract the scaffolds. After incubation, the extracted medium was sterilized by a 0.22 μM PES filter. The filtrated medium was kept at -20°C until usage. The human keratinocyte (HaCaT) and African green monkey kidney (Vero) cell lines were seeded into 96 well plates with a density of 1×10^4 cells per well and incubated at 37°C in an atmosphere containing 5% of CO_2 for 24 h. After culturing for each period, the cells were treated with the extracted medium at 31.25, 62.5, 125, 250, 500, and 1,000 μg/mL for 24 h. Then, the surviving cells were quantified by 3-(4,5-dimethyl thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay. The medium was discarded from each well. The MTT solution (0.5 mg/mL in PBS) was added into each well at around 150 μL and incubated for 1 h to establish cell viability. After that, the formazan crystals were dissolved in 150 μL of pure DMSO, and the absorbance of the solution was measured at 570 nm using a microplate reader (Molecular Devices SpectraMax ABS, USA).

3. Results and Discussion

The microstructure of scaffolds formed by freeze-dry process under SEM image shows in Figure 1. The porous and spongy cells could be observed. The formula of fibroin:gelatin scaffolds at 8:2 reveal some spherical spongy connections, as shown in Figures 1a and 1b, while the formula of fibroin:gelatin scaffolds at 7:3 scaffolds exhibit spongy, spherical, and polygon interconnected evenly shape as shown in Figures 1c and 1d. This result is similar to a previous study by Chamchongkaset et al. [2]. They also found a spongy and uniformly connected fibroin fiber. In addition, Wang et al. [9] studied the structure of scaffolds as a porous spherical lattice that could provide an ideal environment for cell proliferation and better cell growth.

Pore size analysis was performed with the Image J software for the cellular scaffold. The blended scaffold formula ratios of fibroin:gelatin at 8:2 is shown in Figure 2a. The pore size displays approximately 14.14 ± 11.31 micrometers, whereas the pore size for formula ratios of fibroin:gelatin at 7:3 Figure 2b exhibits approximately 19.48 ± 18.71 micrometers. The pore size of these two cell scaffolds was smaller than the scaffolds with a cellulose nanofiber (CNF) to poly (vinyl) alcohol (PVA) ratio at 0.4:0.1 wt% had the average pore size of around 90.71 ± 2.4 micrometers, which was the same as the dermis. The average pore size is approximately 10-15 micrometers, especially the pore size of the scaffold allows the fibroblast cells to penetrate and produce the extracellular matrix (ECM). Moreover, the scaffold with CNF to PVA ratio at 1.4:0.35 wt% had an average pore size of 19.72 ± 3.6 micrometers, similar to the skin cells in the epidermis [10]. Furthermore,

the study of pore size of the agarose-chitosan-collagen type I cell scaffold at a ratio of 3:1:0.5 and 3:1:3 exhibited approximately 170.37 and 241.99 micrometers [10]. Besides, the optimal pore size for bone formation should be 200-400 micrometers [11]. Another previous study of scaffolds fabricated with 75:25 poly (lactic-co-glycolic) acid (PLGA) by the solvent casting particulate leaching technique with NaCl examined the suitable optimum pore size between 150-750 micrometers, exhibiting a pore size comparable to the size of the salt particles used in the fabrication process [12]. From those research, our blended scaffolds between fibroin and gelatin have appropriate pore sizes, allowing the cells to penetrate and produce the extracellular matrix. They might be better for skin tissue engineering.

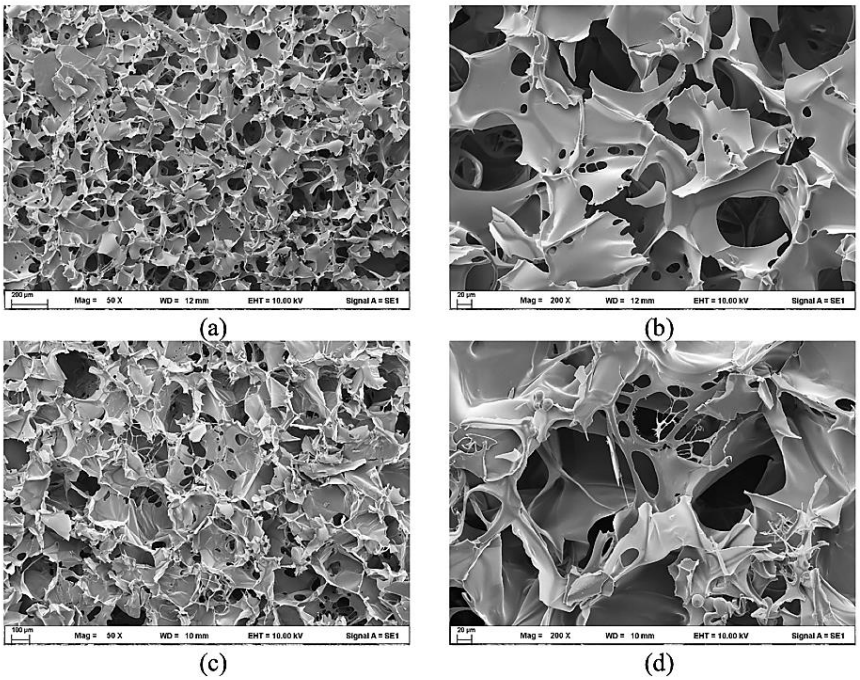

Figure 1. SEM images: (a) scaffold formula ratios of fibroin:gelatin at 8:2 with a scale bar of 50 μm, (b) scaffold formula ratios of fibroin:gelatin at 8:2 with a scale bar of 200 μm, (c) scaffold formula ratios of fibroin: gelatin at 7:3 with a scale bar of 50 μm, (d) scaffold formula ratios of fibroin:gelatin at 7:3 with a scale bar of 200 μm.

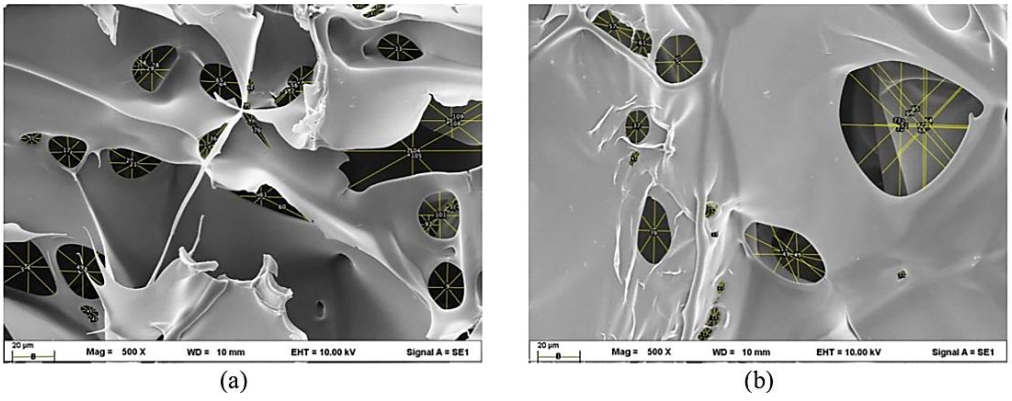

Figure 2. Pore size: (a) scaffold formula ratios of fibroin:gelatin at 8:2 with a scale bar of 20 μm, (b) scaffold formula ratios of fibroin:gelatin at 7:3 with a scale bar of 20 μm.

FTIR analysis was measured to obtain the conformational changes of each scaffold formula (Figure 3a). The spectrum clearly showed the similar chemical structures between fibroin and gelatin that are characteristic of protein absorption. FTIR spectrum of fibroin comprised of amide I (1640 cm^{-1}), indicating the carbonyl (C=O) stretching; amide II (1515 cm^{-1}), showing the vibration on the plane of the N-H bond and C-N stretching; amide III (1236 cm^{-1}) referring to C-N stretching and N-H deformation, and O-H stretching appearing at 3276 cm^{-1} [13-15]. Likewise, the gelatin spectrum presented the main components of amide I, II, and III with a slight shift of wavelength number. In addition, the wavelength number 1399 cm^{-1} indicated the C=O symmetric stretching of the COO-group in the amino acid of gelatin was different from fibroin which has a double peak at 1411 and 1384 cm^{-1} as well as scaffold formula ratios of fibroin:gelatin at 8:2 and 7:3. Therefore, their amino acid and secondary structure were different. The secondary structure of the proteins was estimated thoroughly by the deconvolution and curve fitting of the amide I band [13, 16]. The formula ratios of fibroin: gelatin at 8:2 exhibited higher percentages of random coil structure compared to those of fibroin:gelatin at 7:3 and contained less β-sheet. The results indicated that the increase influenced a slight increase in β-sheet gelatin content (Table 1). This conformation may affect the scaffold's ability to support cells [17].

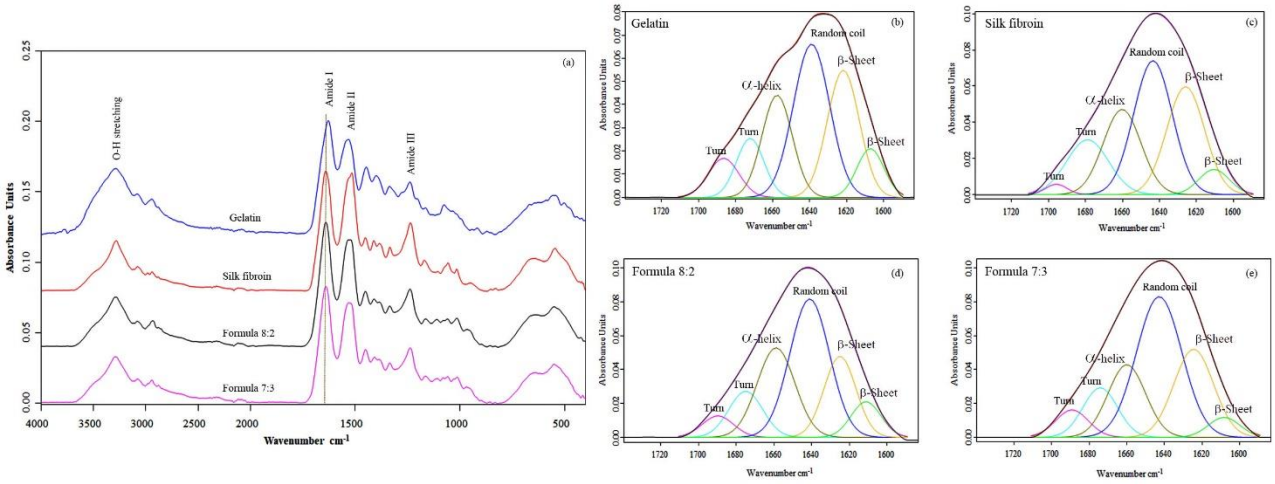

Figure 3. FTIR functional group detection results of gelatin-infused fibroin cell scaffolds (a), The secondary structure of samples determined by Fourier deconvolution of the amide I band of each sample, Gelatin (b), Silk fibroin (c), formula ratios of fibroin:gelatin at 8:2 (d) and formula ratios of fibroin: gelatin at 7:3 (e).

Table 1. The structural conformation ratios in gelatin, silk fibroin, and silk fibroin-gelatin scaffolds derived from deconvoluted amide I FTIR spectra.

Samples	Conformation content of samples [a)]			
	β-sheet	Random coil	α-helix	β-turns
Gelatin	32.96 ± 1.0	34.12 ± 0.5	17.72 ± 0.7	17.20 ± 0.3
Silk fibroin	31.00 ± 1.2	32.69 ± 1.2	19.89 ± 1.5	16.41 ± 1.4
Scaffold 8:2	24.88 ± 0.5	36.58 ± 0.6	21.61 ± 1.2	23.54 ± 0.6
Scaffold 7:3	27.75 ± 1.6	29.47 ± 2.1	21.39 ± 0.5	23.38 ± 1.0

[a)] Values are average ± standard derivation (N=3)

Contact angle measurements were determined to identify the hydrophilic properties of the scaffold. The formula ratios of fibroin:gelatin at 8:2 had a slightly lower contact angle than those of fibroin:gelatin at 7:3. The formula ratios of fibroin:gelatin at 8:2 shows 125.88 ± 9.85 degrees, while the formula ratios of fibroin: gelatin at 7:3 display 130.07 ± 3.72 degrees Figures 4a, 4b, 4c. This might indicate the binding of the N-H group of collagens caused by the polarity of the carboxyl groups. The reduction of polarity with various water contact angles versus time showed that the formula ratios of fibroin:gelatin at 8:2 scaffolds decreased faster than those

of fibroin:gelatin at 7:3 scaffolds, as shown in Table 2. Likewise, a previous study has shown that the water contact angle of silk/collagen (0.25) was 103.27 ± 5.91, silk/collagen (0.50) was 104.27 ± 4.20, silk/collagen (1.00) was 106.55 ± 6.18, and silk/collagen (2.00) was 103.95 ± 2.03 degrees [18]. The angle of contact with water was reduced depending on the measurement time. The high measurement variability is caused by the droplet's location and the scaffold absorption [19].

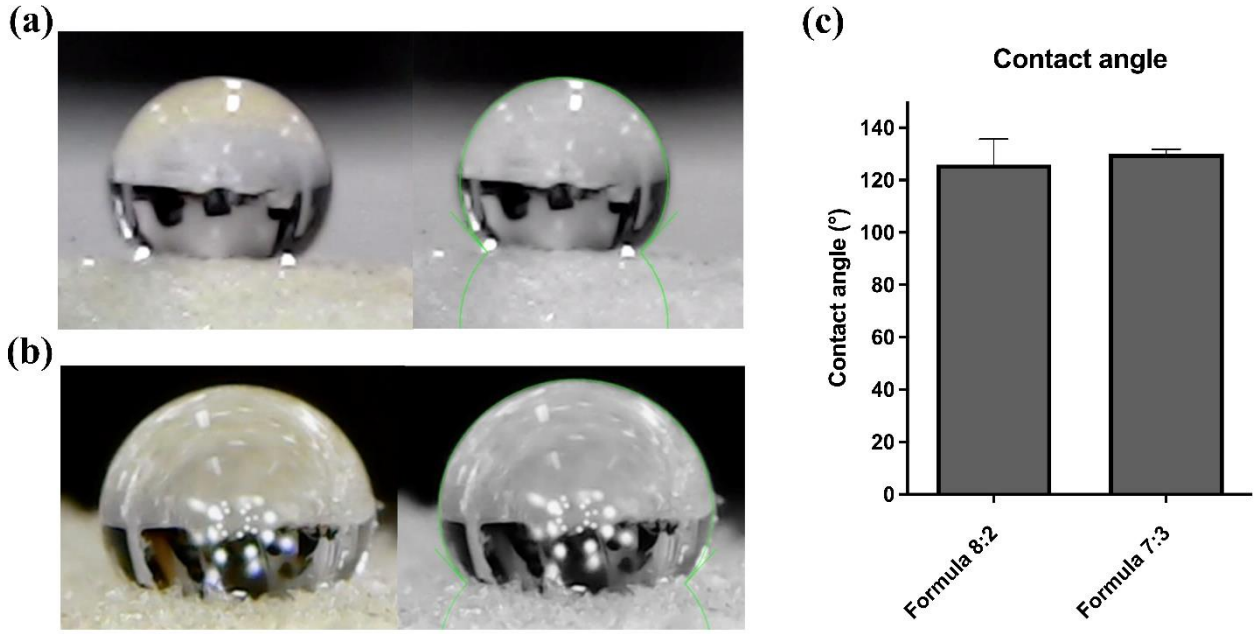

Figure 4. Contact angle: (a) scaffold formula ratios of fibroin:gelatin at 8:2, (b) scaffold formula ratios of fibroin: gelatin at 7:3, (c) a comparison between scaffold formula at 8:2 and 7:3.

Table 2. Water contact angle.

Scaffolds	Water contact angle					
	0 sec	10 sec	15 sec	20 sec	25 sec	30 sec
formula 8:2	125.88 ± 9.85	110.72 ± 7.80	95.29 ± 0.85	82.75 ± 5.84	67.22 ± 7.94	51.34 ± 5.11
formula 7:3	130.07 ± 3.72	109.22 ± 6.14	84.73 ± 5.35	72.74 ± 10.57	64.08 ± 7.84	49.79 ± 6.62

The crystal structure of scaffolds was studied using Wide Angle X-ray scattering. The Gaussian function was used to fit the diffraction peaks, and the Lorentz function was used to hold the amorphous background to estimate crystallinity, as shown in Figure 5. The WAXS pattern of the two scaffolds demonstrated diffraction peaks at 7.29°, 19. 50°, and 39.38°. The crystallinity percentage of formula ratios of fibroin: gelatin at 8:2 was 39.039%, whereas the formula ratios of fibroin: gelatin at 7:3 was 41.936%. For the WAXS result, the crystallinity of formula ratios of fibroin:gelatin at 7:3 was slightly higher than that of fibroin: gelatin at 8:2. The gelatin induced fibroin conformational conversion from random coil to beta crystal [20]. Therefore, the presence of gelatin influences gelatin-blended gelatin-blended fibroin scaffold crystallization. The characteristic of these two scaffolds was almost the same as those of the β-sheet crystalline structure (silk II) of silk fibroin [21-22] due to the lower ratio of gelatin.

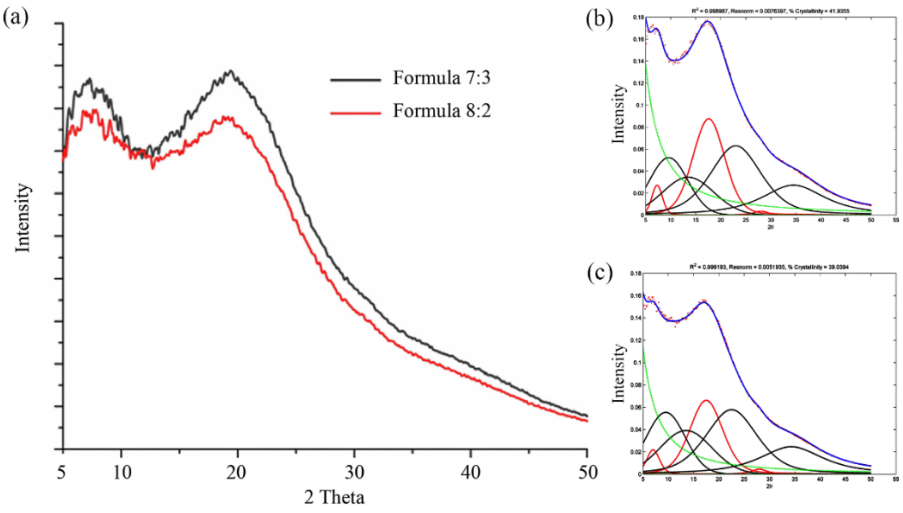

Figure 5. The WAXS pattern of scaffolds (a), fitting curves of formula ratios of fibroin:gelatin at 7:3 (b) and fitting curves of formula ratios of fibroin:gelatin at 8:2 (c).

The biocompatibility test was measured to evaluate the cytotoxicity on regular cell lines. Vero cells were treated with scaffold formula ratios of fibroin:gelatin at 8:2 with various concentrations of 62.50-1000 μg/mL. Cell viability percentages were in the range of 96.31-103.22%. Similarly, for scaffold formula ratios of fibroin:gelatin at 7:3 (62.50-1000 μg/mL), the cell viability percentages were 106.38, 104.28, 108.54, 97.42, and 97.65%, respectively. The results show that both scaffolds at different concentrations do not produce toxicity to Vero cells and unchanged cell viability compared to the control, as shown in Figures 6a and 6b. HaCaT cells viability after treatment with the scaffold formula ratios of fibroin:gelatin at 8:2 (62.50-1000 μg/mL) had cell viability percentages around 104.86, 111.71, 109.58, 113.23, and 119.90%, respectively, while the formula ratios of fibroin:gelatin at 7:3 displayed cell viability percentages around 99.62, 101.02, 113.16, 114.38 and 101.36%, respectively. The result revealed that the scaffolds at different concentrations were also not toxic to HaCaT cells or did not decrease cell viability due to cytotoxicity on the Vero cell, as shown in Figures 6c and 6d. These results confirmed that the scaffold could be applied to the human body for medical application and tissue engineering. Similarly, the previous study cultured the fibroblast cells on agarose-chitosan-collagen type I cell scaffolds at a ratio of 3:2:0.5, showing that fibroblast cells could grow and multiply well in the scaffold. Thus, this scaffold was not toxic to fibroblast cells [10]. An additional study was tested on gelatin-fibroin-chitosan-produced cell scaffolds. It was found that all ratios of scaffolds had a percentage of viability of more than 80%, indicating that scaffolds had no cytotoxicity [23]. Therefore, our results confirmed the scaffolds are biocompatible with skin (HaCaT) and kidney (Vero) cell lines, indicating that they can be used in the human body and might restore damaged skin or internal organs for tissue engineering applications.

4. Conclusions

This study investigated the fabrication of blended scaffolds between fibroin and gelatin by the freeze-drying process for tissue engineering. The porous scaffold by SEM images is suitable for penetrating fibroblast cells and producing the ECM. The chemical functional structure of scaffolds revealed an amide group inside the scaffold, exhibiting a lattice-like crystalline form. The microstructure of the blended scaffolds had porous and connected evenly, which was a suitable environment for cell proliferation. Furthermore, these scaffolds were non-cytotoxic and biocompatible with Vero and HaCaT cells. Our results demonstrated that fibroin/gelatin scaffolds exhibited good biological properties and could be supported because they might apply to the human body. Hence, our scaffolds are a strong candidate biomaterial appropriate for manufacturing as innovative skin tissue engineering products for further applications.

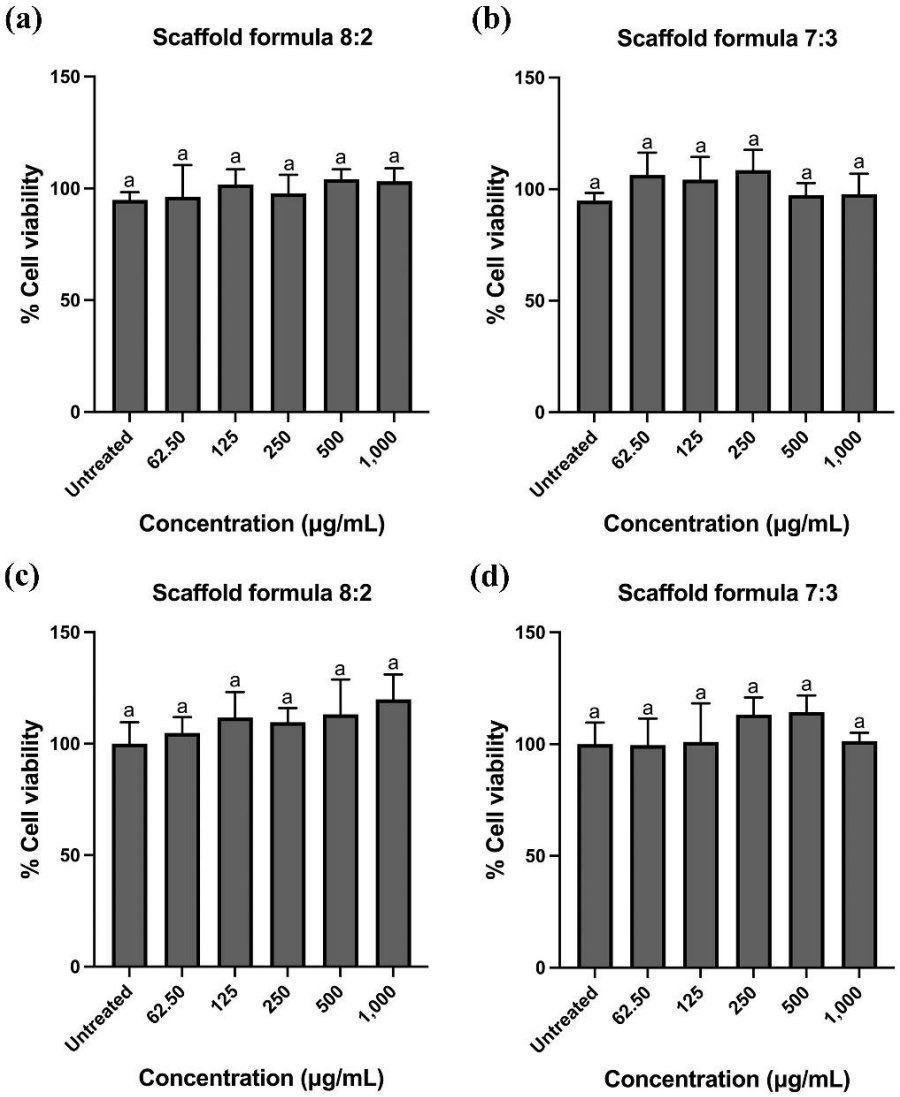

Figure 6. Cell viability with MTT assay: (a) cell viability analysis of Vero cells on scaffold formula ratios of fibroin: gelatin at 8:2, (b) Vero cells on scaffold formula ratios of fibroin:gelatin at 7:3, (c) cell viability analysis of HaCaT cell on scaffold formula ratios of fibroin:gelatin at 8:2, (d) HaCaT cell viability on scaffold formula ratios of fibroin:gelatin at 7:3. The data were displayed as means ± S.D. of triplicate determinations. Different letters on the top of each bar indicate statistically significant differences ($p < 0.05$).

5. Acknowledgements

This study was supported by the Faculty of Engineering, Burapha University. The authors gratefully acknowledge the support of the Synchrotron Light Research Institute (Public Organization), Nakhon Ratchasima, Thailand, for a wide-angle synchrotron X-ray scattering instrument.

Author Contributions: Conceptualization, S.P.; methodology, K.N., and S.C.; formal analysis, K.N., S.C., P.C., and S.P.; data curation, K.N.; writing-original draft preparation, K.N.; writing-review and editing, K.N., S.C., P.C., and S.P.; supervision, S.P. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Conflicts of Interest: The authors declare no conflict of interest.

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