Fibroin and Spidroin Thin Film to Support the Attachment and Spread of Human Dermal Fibroblast: The Potency of Skin Tissue Engineering

Safira Meidina Nursatya[1], Anggraini Barlian[1]*, Hermawan Judawisastra[2], Indra Wibowo[1] & Hutomo Tanoto[2]

[1]Biotechnology Study Program, School of Life Sciences and Technology, Institut Teknologi Bandung, Jalan Ganesha 10, Bandung 40132, Indonesia
[2]Materials Engineering Study Program, Faculty of Mechanical and Aerospace Engineering, Institut Teknologi Bandung, Jalan Ganesha 10, Bandung 40132, Indonesia
*E-mail: aang@sith.itb.ac.id

Abstract. This study aimed to determine the characteristics of scaffolds made of fibroin from *Bombyx mori* and spidroin from *Argiope appensa* in supporting the attachment and proliferation of HDF cells on the scaffolds. Thin-film scaffolds were made using the solvent casting technique, where the scaffold is an amalgamation of fibroin, spidroin, PVA, and glycerol. HDF cells were grown on DMEM medium with 10% FBS and 1% antibiotic-antimicotic. Characterization of the scaffolds was performed by using ATR-FTIR, swelling test, contact angle measurement, tensile test, biodegradation, MTT and SEM. The results of the ATR-FTIR analysis showed that the scaffolds contained fibroin, spidroin, PVA, and glycerol. Swelling and contact angle tests showed that all scaffold combinations were hydrophilic. Mechanical properties and in vitro biodegradation tests showed no significant difference among the scaffold combinations. MTT testing showed that all scaffolds could facilitate the attachment of fibroblasts and showed increased viability from day 1, 3, and 5. Scanning electron microscopy showed that the cells in the 70% fibroin and 10% spidroin scaffold had the best cell morphology and the best combination for potential application in skin tissue engineering.

Keywords: *Argiope appensa; Bombyx mori; biomaterial; cell attachment; cell morphology.*

1 Introduction

Burns are a serious health problem. Skin defects caused by burning can affect an individual physically and psychologically [1]. According to one study conducted in Indonesia (2015), 66.3% of 104 patients with severe burns died [2]. In addition to the limited availability of donors, the skin graft method cannot be applied in cases of severe wounds that cause skin tissue loss of up to 50 to 60% of the total body surface area (TBSA) [3].

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Aside from donated skin, there are also collagen-based skin substitutes. Currently, several collagen-based skin substitutes, such as Dermagraft and Apligraf, are widely used. However, they have downsides, such as low mechanical properties and being expensive [1]. Human skin has a tensile strength of about 2.0 to 16.0 MPa [4] and tensile stiffness values in the range of 15.0 to 150.0 MPa [5]. Therefore, the scaffold for human skin must be within this range to demonstrate good mechanical strength and ensure stability for angiogenesis, the lymphatic system, nerves, and other structures [6]. Tissue engineering can be used as a solution, by engineering a scaffold to fulfill these requirements, thus making it applicable as a support material during the regeneration process of cells and tissues.

In skin tissue engineering, human dermal fibroblast cells (HDF) are most often used as the source of cells. HDF cells are among the cells that make up skin tissue found in the dermis. They can differentiate into myofibroblasts and play an important role in the wound healing process [7].

Silk is a biological polymer composed of various polypeptides. Silk fibroin is produced by several types of silkworms and silk spidroin is produced from spider webs. Fibroin has unique physical and chemical characteristics and has excellent mechanical characteristics [8]. Spidroin obtained from spider webs is known for its excellent mechanical strength and non-toxicity [9]. In several studies in the manufacturing of biomaterials for scaffolds, spidroin or fibroin silk was used because both have high strength (500-972 MPa), do not trigger an immune response (low immune response), and are non-toxic, biocompatible, and biodegradable [10].

So far, one spidroin-based skin tissue engineering scaffold has been developed, from *Nephila sp*. *Argiope appensa* is a specific type of spider that can be found in Indonesia. This spider is speculated to have arginine-glycine-aspartate (RGD), which is a specific amino acid sequence that can help any cell to proliferate and differentiate faster [11].

In addition to fibroin and spidroin, synthetic materials are needed in the engineering of skin tissue so that the scaffold can fulfill a number of specific requirements. PVA (polyvinyl alcohol) is a hydrophilic synthetic polymer (containing -OH). This polymer has good biocompatibility and biodegradability. The addition of PVA will help the scaffold to maintain its mechanical stability and flexibility, and adjust its degradation kinetic [12-14]. Glycerin, or glycerol, is well-known for its plasticizing properties, where controlling the addition of glycerin will affect the strain and stiffness of the scaffold [15]. Moreover, the use of glycerin is known to prevent the scaffold from dissolving, because glycerin helps the PVA and fibroin/spidroin to form crosslinking chains.

The lack of mechanical properties and high cost of commercial skin graft products are a reason to developed suitable substitutes. Scaffolds from green natural sources are believed to have good compatibility with the human body. Silk fibroin was chosen as the main component because of its abundant existence, while small amounts of PVA and glycerin were added only to adjust the mechanical properties and the stability of the scaffold. Addition of spidroin from *Argiope appensa* has the purpose of speeding up the HDF cell proliferation to shorten the healing process.

2 Materials and Methods

2.1 Material

The materials used in this study were: silkworm cocoons (*padepokan dayang sumbi*), spider silk, PVA (Polyvinyl Alcohol) (Sigma-Aldrich Mw~130,000), glycerol (Pudak Scientific), $NaHCO_3$ (Sigma-Aldrich), $CaCl_2 2H_2O$ (Pudak Scientific), formic acid (Merck), HDF cells, ethanol (Merck), DMEM (Dulbecco's Modified Eagle's Media) (Sigma-Aldrich), PBS (phosphate-buffered saline) (Gibco), FBS (fetal bovine serum) (Gibco), ABAM (Antibiotic-Antimycotic)(Gibco), MTT(3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) (Sigma-Aldrich), Trypsin-EDTA and protease xiv *Streptomyces griseus* (Merck Sigma-Aldrich).

2.2 Thin-film Scaffold Fabrication

Thin films were made using the solvent casting method. The manufacture of thin films from fibroin began with degumming and cleaning the sericin membrane on the cocoons by heating the cocoons in 0.05 wt% $NaHCO_3$ solution for one hour, then drying them for 24 hours. Furthermore, the fibroin was dissolved for 6 hours in $CaCl^2$/et-OH/H2O = 1:2:8 (mass ratio). After that, dialysis in deion was conducted for three days, with water changes every 2-6 hours, followed by freeze drying. The fibroin and spidroin dissolved in formic acid, PVA and glycerin were homogenized for 3 hours and poured into a mold. The mold was placed in a fume hood to evaporate the solvent for 24 hours [15,17,18].

2.3 Human Dermal Fibroblast (HDF) Culture

In this study, the cells used were human dermal fibroblast cells obtained from the primary culture of the foreskin (prepuce). The cells used passage 19 cells. The cells were maintained in DMEM (Dulbecco's Modified Eagle's Media) culture medium, to which 10% FBS and 1% antibiotic-antimicotic were added. Cells were grown at 37 °C with 95% humidity and 5% CO_2. The culture medium was changed every two to three days [19,20].

2.4 Structural Characterization

FTIR (Fourier transform infrared) spectroscopy was performed to analyze the structure of the scaffolds. Attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) was performed to assess the secondary conformation of the scaffolds. ATR-FTIR measurements were performed using a FTIR broker alpha II. ATR-FTIR spectroscopy was recorded between 1000 cm^{-1} and 4000 cm^{-1}.

2.5 Contact Angle

The hydrophilicity of the film was determined by analyzing the angle of water droplets formed between the different liquid/solid film interfaces using the drop sessile method. The volume of water droplets was dripped by 4 μl [21].

2.6 Water Absorption Test

The water absorption (%) of the film was calculated by the following equation:

$$(\%) = (Ws - Wd)/Wd \times 100 \quad (1)$$

where *Ws* is the weight of the sample after immersion and *Wd* is the weight of the dry sample. The dry sample weight was measured directly. The sample weight after immersion in PBS at 37 °C for 48 hours was measured after drying the film surface [22].

2.7 Mechanical Properties

The mechanical properties of the thin films were characterized using a Universal Testing Machine according to ASTM D 882. A sample size (70 × 10 mm) was pre-conditioned and mounted between the machine handles, which were pulled at a speed of 1 mm/min [23].

2.8 Biodegradation

Sterile samples were cut into specific sizes and weighed. The samples were incubated in 96 well plates in PBS with protease XIV (2 mg/mL). This well plate was placed in an incubator set at 37 °C. The samples were observed with intervals of 3, 7, 10, and 14 days. They were then removed, surface dried, and weighed. The percentage of biodegradation was calculated using the following equation:

$$(\%) = Wo - Wt/Wo \times 100 \quad (2)$$

where *Wo* is the initial dry mass and *Wt* is the final mass measured after incubation [24].

2.9 Collective Information and Protocol Isolation of HDF Cell Culture

Cell cultures obtained from circumcision, with foreskin tissue as a source for HDF obtained from the Seno Medika clinic. The foreskin tissue obtained from the clinic was sterilized using 10% povidone-iodine solution, alcohol 70%, phosphate-buffered saline (PBS) 1x solution. After that, adipocytes and necrotic tissue were removed using dispase (type II, 2.4 mg/mL; Sigma) and collagenase (type I, 220 units/mg, Gibco). HDF cells were grown from explants and subcultured.

2.10 MTT Assay

The experiment was carried out on 96-well plates. HDF cells were grown 2000 cells/well and allowed to attach for 4 hours. Then, the medium was added to each well, and the plates were incubated for 1, 3, and 5 days at 37 °C. At the end of the experiment, cell viability was assessed by MTT test (0.5mg/mL final concentration) [25].

2.11 SEM (Scanning Electron Microscope)

HDF cell morphology on the thin-film surface was observed by SEM. HDF was seeding on a scaffold for three days at 37 °C; 5% CO_2. The samples were fixed with 100 µl 2.5 glutaraldehyde in 0.1 M cacodylate buffer, incubated overnight at 4 °C, dehydrated with alcohol series, and dried using HMDS overnight. The samples were then plated with gold and observed under SEM (SU 3500; Hitachi, Krefeld, Germany; Center of Advanced Science ITB).

3 Results and Discussion

3.1 Fibroin/Spidroin/PVA/Glycerin Thin-film Scaffolds

In this study, six scaffold combinations were successfully made from fibroin/spidroin/PVA/glycerin. The concentrations of the materials used were 10% w/v fibroin, 8% w/v spidroin, 10% w/v PVA, and 10% w/v glycerin. Each combination was made with a different percentage of fibroin and spidroin volume, while the percentage of PVA and glycerin was the same in each scaffold combination. There were six scaffold combinations in this study, namely 80F0SPG (80% fibroin, 10% PVA, 10% glycerin), 78F2SPG (78% fibroin, 2% spidroin, 10% PVA, 10% glycerin), 76F4SPG (76% fibroin, 4% spidroin, 10% PVA, 10% glycerin), 74F6SPG (74% fibroin, 6% spidroin, 10% PVA, 10% glycerin), 72F8SPG (72% fibroin, 8% spidroin, 10% PVA, 10% glycerin), and 70F10SPG (70% fibroin, 10% spidroin, 10% PVA, 10% glycerin) (Figure 1). The scaffold produced in this study was transparent, yellowish-white, flexible, and

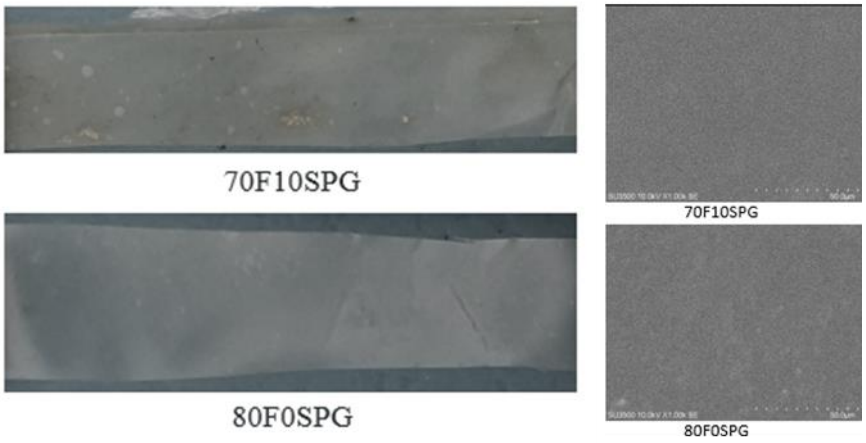

Figure 1 (a) Sample of fibroin/spidroin/PVA/glycerin thin-film scaffold (b) SEM images of scaffold (the number in the name of the sample shows the percentage to volume ratio, F = fibroin, S = spidroin, P = PVA, and G = glycerol).

had a thickness of 0.16 ± 0.10 mm. The thickness of the thin-film scaffold was not much different from the thickness of the epidermis in human skin, which ranges from 0.10 to 0.20 mm [26].

3.2 Structural Characterization

The sample is an amalgamation of fibroin, spidroin, PVA, and glycerol (Figure 2). The differences between the FTIR result of pure spidroin and the mixture were at wavenumbers 3248, 2936, and 1083 cm^{-1}. These areas showed characteristics of OH stretching (3248 cm^{-1}), CH_2 asymmetric stretching (2936 cm^{-1}), and C=O stretching and OH bending (1083 cm^{-1}), which were originally from the PVA and glycerol function group [27].

In sum, the addition of silk spidroin neither added nor created new functional groups in the fibroin mixture. However, the addition did increase some of the wavenumber areas, i.e. β-sheet region at 1630, 1530, and 1240 cm^{-1}, random coil at 1650, 1550, and 1230 cm^{-1}, and α-helix at about 1655 cm^{-1} [11].

3.3 Contact Angle

Characterization of a material's contact angle is carried out to determine its hydrophilicity [22]. The contact angle data results in this study ranged from 57.18 to 69.78 (Figure 3). According to [17], a material can be classified as hydrophilic if it has a contact angle of <90°. Thus, it can be inferred that all thin-film scaffold

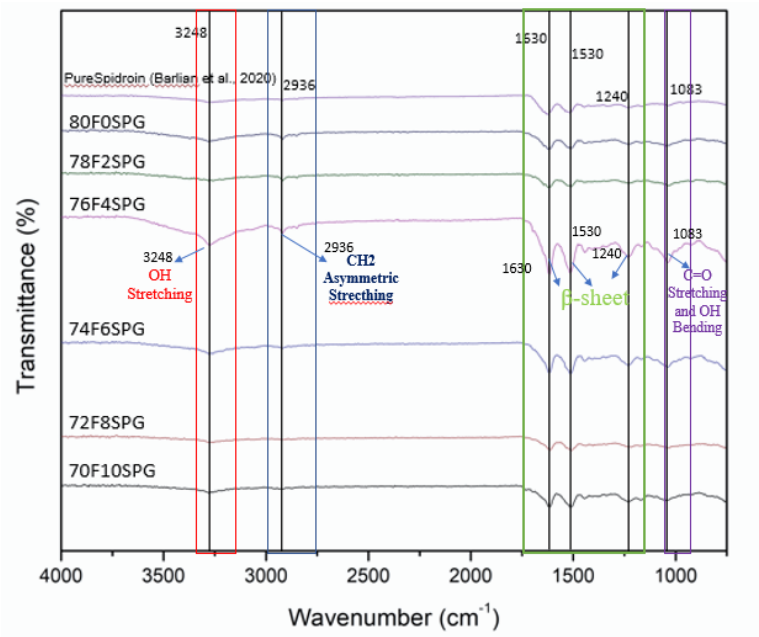

Figure 2 FTIR fibroin/spidroin/PVA/glycerin thin-film scaffold (the number in the name of the sample shows the percentage to volume ratio, F = fibroin, S = spidroin, P = PVA, and G = glycerol).

combinations produced in this study were hydrophilic. The results of the calculation of the contact angle from another study that also used fibroin as thin film had a contact angle value of 71°, while thin film modified with the addition of dextrose material had a contact angle value of 60°. These results from [22] are in line with those of the present study, which showed that based on the value of the contact angle the material could be classified as hydrophilic. The *Nephila sp* spidroin thin-film scaffold in Ref. [17] showed that based on its contact angle (54°) it could also be classified as hydrophilic.

The hydrophilicity of the material is important to know because a good scaffold must be able to facilitate cells to attach and proliferate. It is known from previous research that very hydrophilic or highly hydrophobic materials do not support cell attachment and proliferation [28]. A scaffold surface with moderate hydrophilicity (40-70°) is known to be the most optimal for protein absorption and cell response [29]. Based on the results of the present study, the resulting scaffold was in the category of moderate hydrophilicity. Thus, it is optimal and highly supports cell growth for application as a tissue engineering scaffold.

The ingredients of the scaffold play a role in the hydrophilicity of the resulting sample. Fibroins are known to have hydrophilic amino acids and carboxyl groups

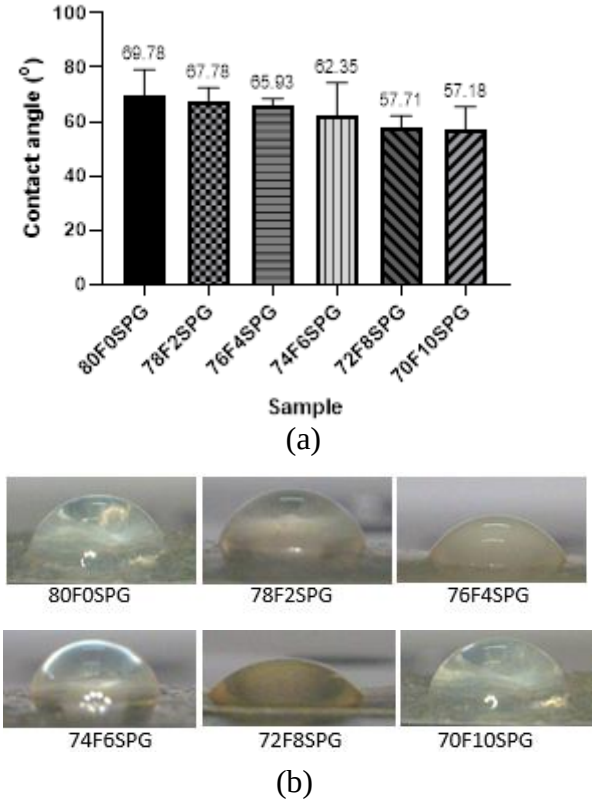

Figure 3 (a) Contact angle of fibroin/spidroin/PVA/glycerin thin-film scaffold, (b) optical images of the contact angle (the number in the name of the sample shows the percentage to volume ratio, F = fibroin, S = spidroin, P = PVA, and G = glycerol). The data are mean ± SD.

that can increase hydrophilicity [30]. In the spidroin *Argiope appensa* there is an amino acid, RGD sequence [11]. The contact angle data shows that a sample containing more spidroin is more hydrophilic. This is because amino acid glycine is classified as a hydrophilic amino acid [31]. Apart from fibroin and spidroin, PVA and glycerin also play a role in scaffold hydrophilicity. PVA is a hydrophilic material [32], while glycerol is known to be included in polyol compounds [33].

3.4 Water Absorption Test

Apart from characterizing the contact angle, a water absorption test was also carried out. In skin tissue engineering, water absorption testing is important, because when a scaffold is applied to injured skin there will be exudate, which if left unchecked can become a place for bacteria to live [22]. In this study, the water absorption of the 70F10SPG sample in the range of 37 to 61% was higher than

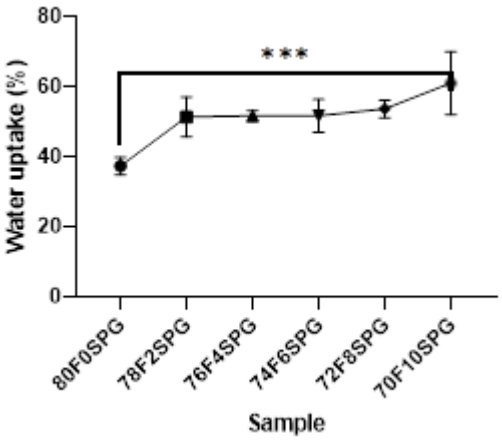

Figure 4 Water uptake of fibroin/spidroin/PVA/glycerin thin-film scaffold (the number in the name of the sample shows the percentage to volume ratio, F = fibroin, S = spidroin, P = PVA, and G = glycerol). The data are mean ± SD with a significance marker *** ($p < 0.001$).

that of the other scaffolds (Figure 4). Consistent with Ref. [22], the percentage of silk fibroin was 15%, while fibroin with the addition of dextrose material had a water absorption percentage of 32 to 63%. Compared with the scaffold in Ref. [17], the spidroin thin-film scaffold had a water absorption percentage of 53.63%. The results here are not much different from those of other studies. The increased percentage of water absorption can be related to the increase in hydrophilic groups present in the scaffold [18]. This is also supported by the contact angle data, which shows that the 70F10SPG sample had the lowest (most hydrophilic) contact angle.

3.5 Mechanical Properties

Among the various characterizations of existing materials, the mechanical properties are important to consider in scaffold fabrication. Mechanical properties are identified so that they can be implanted in the desired tissue [34]. The most common mechanical tests used to evaluate scaffolds are tensile tests and compressive tests. Each application in tissue engineering requires a different range of mechanical properties depending on the tissue [6]. Because each scaffold application requires a different mechanical property, different tests are used. A scaffold that is applied to the skin requires a tensile test [35]. The tensile test analysis included three aspects: tensile strength, tensile strain, and tensile stiffness.

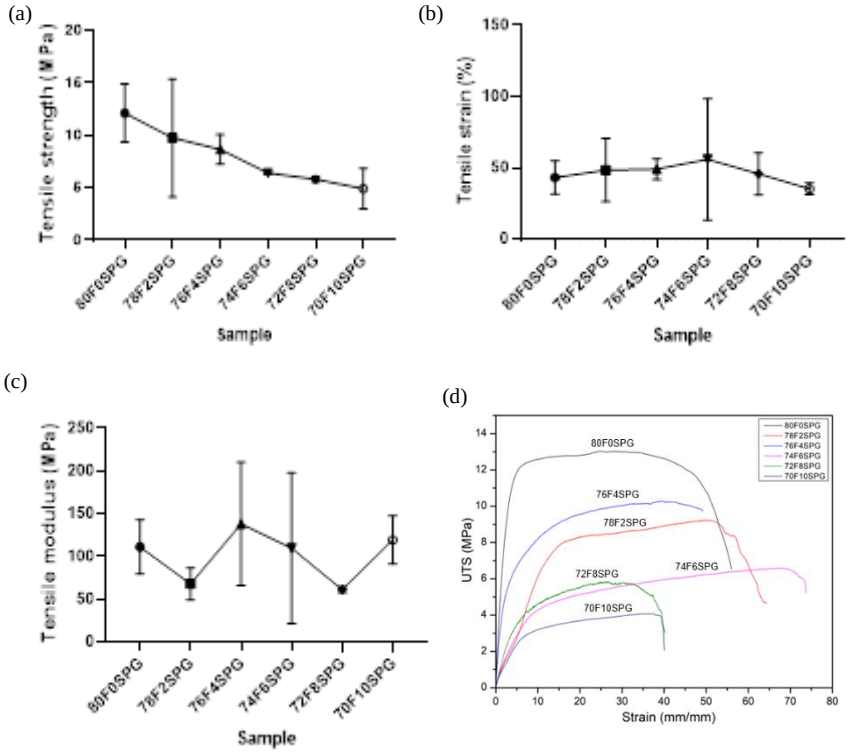

Figure 5 Mechanical properties of thin-film scaffold (the number in the name of the sample shows the percentage to volume ratio). F = fibroin, S = spidroin, P = PVA, and g = glycerol. The data are mean ± SD: (a) tensile strength; (b) tensile strain; (c) tensile modulus; (d) stress-strain curves.

The test showed that the scaffold's tensile strength was 4 to 12 MPa (Figure 5a). The fibroin concentration used in this study was 10% w/v, while the spidroin concentration was 8% w/v. The samples that did not contain spidroin had the highest tensile strength, an outcome that may be due to differences in the concentrations of fibroin and spidroin used [36]. Such differences in concentration affect the weight of the silk used; the 80F0SPG sample contained more silk than the other samples. The tensile strength of the scaffold in this study had a higher strength than chitosan-based skin scaffold, which has a strength of 1.4 to 1.8 MPa [37]. Real human skin tissue is known to have a tensile strength of 2 to 16 MPa [4]. The thin-film scaffold in this study can be applied in skin tissue engineering because its tensile strength meets the strength criteria of human skin.

The percentage of tensile strain on the thin-film scaffold in this study had a percentage of 35 to 55% (Figure 5b). When compared to findings from other thin-

film studies using fibroin scaffolds, this thin film had a higher tensile strain percentage and thus was more flexible. In Ref. [38], the percentage of thin-film tensile strain was 7 to 12%. The reason for this difference is that Ref. [38] did not use glycerol. Thin films that use glycerol can have increased flexibility [39]. In real human skin, the tissue has a tensile strain of 35 to 115% [5]. The tensile strain possessed by thin-film scaffolds in this study is consistent with the tensile stretch range of real human skin.

The tensile modulus value of this thin-film scaffold ranged from 61 to 138 MPa (Figure 5c). The tensile modulus of human skin is known to be 15 to 150 MPa [40]. Thus, the tensile modulus of the scaffold falls within the tensile modulus range required by human skin. The mechanical properties data of the thin-film scaffold show that it can be applied in skin tissue engineering because the values of tensile strength, tensile strain, and tensile modulus are inside the criteria range of human skin.

3.6 In Vitro Biodegradation

Protease XIV enzyme was used for the in vitro biodegradation test. Protease XIV is a bacterial protease cocktail that has been frequently used in silk degradation [41]. In this study, the thin-film scaffold was completely degraded on days 10 and 14. On the 3rd day, the thin-film scaffold degraded as much as 20 to 36%, and on the 7th day as much as 57 to 83% (Figure 6).

In the studies conducted in [24] on collagen hydrogel samples used for burns, the samples completely degraded on day 10, the same as in this study. Rapid

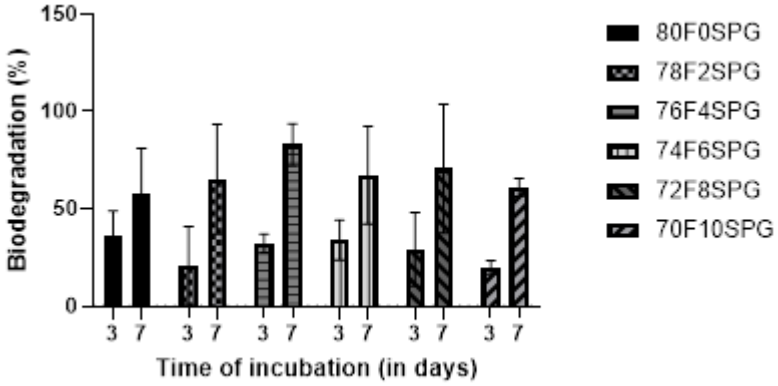

Figure 6 Percentage of thin-film scaffold biodegradation for different fibroin and spidroin compositions (the number in the name of the sample shows the percentage to volume ratio). F = fibroin, S = spidroin, P = PVA, and G = glycerol. The data are mean ± SD.

degradation of the scaffold is caused by the hydrophilicity of the material [42]. Among other enzymes, proteases are known to be more aggressive than alpha-chymotrypsin or collagenase, causing a high degradation rate [43].

3.7 MTT Assay

The metabolic activity of cells seeded on the scaffolds was measured by the MTT test ([3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide]). The MTT test determines the activity of cellular metabolism based on the color change of MTT from yellow to purple (formazan crystals). The color change is caused by mitochondrial dehydrogenase. The optical density (OD) value is related to cell viability; the higher the OD value, the higher the cell viability [25].

All thin-film scaffold samples showed increased cell viability with increasing time. The graph shows that the 70F10SPG sample had the highest cell viability compared to the other samples (Figure 7). This finding is in line with Ref. [11], which found that the scaffold sample containing 10% spidroin had the highest cell viability compared to the other investigated scaffold samples. The high cell viability in the sample containing the most spidroin (70F10SPG) could be caused by the presence of sequential RGD in spidroin, as it is known that the *Argiope appensa* spidroin has sequential RGD [11]. RGD (arginine-glycine-aspartate) is not present in *Bombyx mori* fibroin [44]. The RGD sequence is a binding site for integrins. Integrins are receptors on the cell surface [45,46].

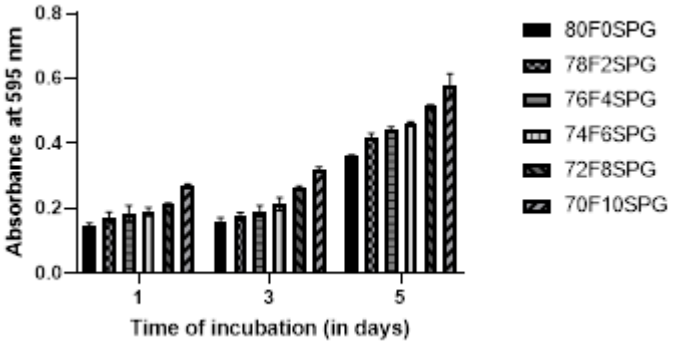

Figure 7 Cell viability of thin-film scaffolds with different fibroin and spidroin compositions (the number in the name of the sample shows the percentage to volume ratio). F = fibroin, S = spidroin, P = PVA, and G = glycerol.

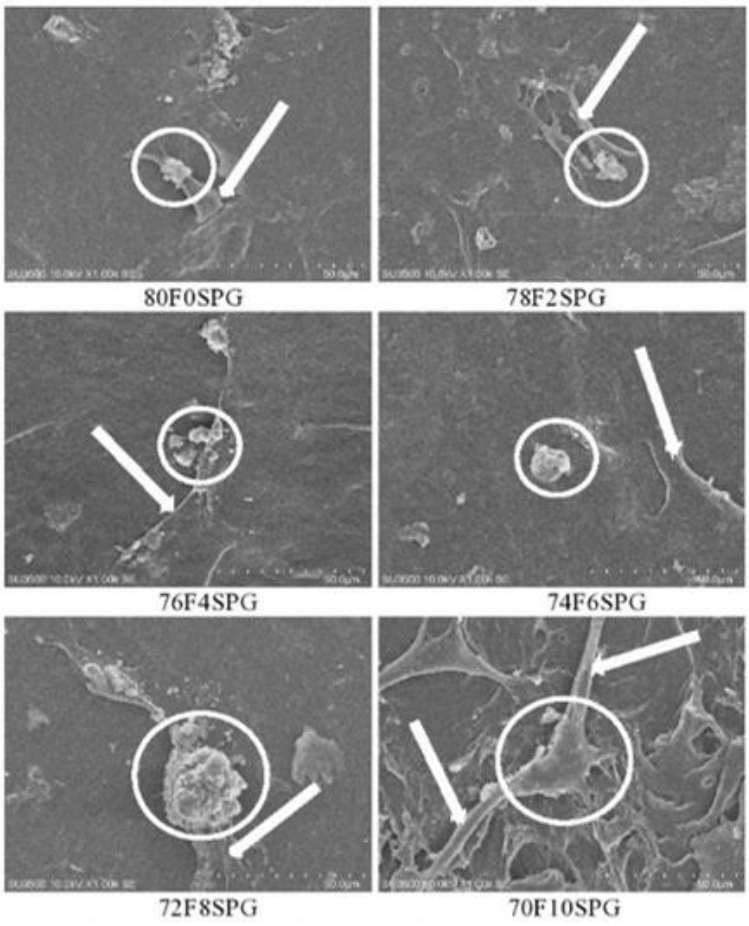

Figure 8 Cells in thin-film scaffolds with different fibroin and spidroin compositions (the number in the name of the sample shows the percentage to volume ratio). F = fibroin, S = spidroin, P = PVA, and G = glycerol. The presence of fibroblasts in the SEM results is indicated by white circles. The white arrows indicate cell filopodia.

3.8 Scanning Electron Microscopy

It can be seen in Figure 8 that fibroblast cells could attach to all thin-film scaffold samples. The SEM analysis results in the images show the differences in cell morphology. In the 80F0SPG sample, the cells were still round and the filopodia were short. In the 70F10SPG sample, the fibroblasts appeared to have spread, and the filopodia were longer. Cells are indicated by a white circle and filopodia are indicated by white arrows. According to Ref. [7], cell spreading is important for wound healing. Cell spreading indicates that the cells are actively secreting collagen, differentiating into myofibroblasts, and secreting GF (growth factor)

cytokines. The 70F10SPG thin film in this research supports HDF cell attachment and spreading, as was also measured by the MTT assay (Figure 7) and HDF cell morphology.

4 Conclusion

Thin-film scaffolds were successfully made by the combination of fibroin/spidroin ranging from 80% F/0% S to 70% F/10% S with 20% of the rest is 10% PVA and 10% glycerol. The thin-film scaffolds fully degraded in 10 to 14 days. All scaffolds showed promising results based on the characterizations that were performed. The water uptake and the contact angle test showed that all scaffolds could absorb water at around 37 to 61% and had moderate hydrophilic behavior, which is preferable in terms of protein absorption and cell response. From a mechanical perspective, all scaffolds showed ranges of tensile strength, modulus, and strain that are within the mechanical properties of human skin tissue. SEM characterization determined that 70% F/10% S is the best candidate for skin tissue engineering, since the cell morphology showed more spreading of cells. This spread pattern is a sign that 70% F/10 %S will make cells able to differentiate faster, which expedites the skin healing process.

The challenge of this research was in the method of dissolving the spidroin into liquid since no stable method has been published, especially for dissolving spiderweb into bioink. Another challenge is finding a chemical to make the amalgamation of fibroin and spidroin become miscible. Nevertheless, the result of this research is promising since the thin film scaffolds that were added with partially successful dissolved spidroin showed more spreading of cells than the scaffold with 0% spidroin. With a proper dissolving and mixing process of spidroin into a scaffold, the result is expected to be significantly improved.

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