Fabrication and characterization wound dressing form silk fibroin/curcumin film incorporating natural oil

Vu Nguyen Doan[1,2*] and Ha Le Bao Tran[1,2]
1. University of Science, Ho Chi Minh City, VIETNAM
2. Vietnam National University, Ho Chi Minh City, VIETNAM
*dnvu@hcmus.edu.vn

Abstract
Nowadays, silk fibroin is widely applied in many biomedical researches including the potential research field in fabricating wound dressings. Curcumin which is traditional herbal, has many suitable features for treatment of skin loss lesions such as antiinflammatory, antibacterial, antifungal, especially reducing formation of scars and improvable wound healing. Furthermore, the incorporation of natural oils improves the physical properties of the fibroin/curcumin film. In this research, silk sericin is degummed by high temperature alkaline solution to collect fibroin fibers. Fibroin fibers and curcumin flour are dissolved by 98% formic acid supplement calcium chloride. Then coconut oil is added to the final mixture with concentrations 0.4%, 0.6% (v/v) respectively.

Fabricated fibroin/curcumin films (FCF) are evaluated with properties such as morphology surface, mechanical tensile, water uptake, water vapor transmission rate, biodegradation capability, pH changing, preventing bacteria penetration capability and in vitro cytotoxicity test. All results showed that FCFs prepared with different concentrations of coconut oil met all the required properties of wound dressings. This research is the initial step in creating the foundation and direction for the development of commercial silk wound dressings.

Keywords: Silk, fibrion fiber, wound dressing, curcumin, wound healing process.

Introduction

Burn, a skin loss lesion, is the worldwide public health affair, causing about 265.000 deaths annually. Most burn cases are present in low and middle-income countries with nearly half occurring in the Southeast Asia Region[15]. Wound dressings help to create a suitable moist environment within the wound and act as a barrier against external factors such as dust and bacteria, increase water and vapor permeability, allow gas exchange and improve epithelialization. Therefore, wound dressings are often used in the treatment of burn wounds[16,22]. Silk fibroin (SF), a specific natural macromolecule is spun by the silkworm *Bombyx mori*, is widely used in the textile and sewing thread industries.

Recently, many researchers suggest SF as a potential biomaterial of biomedical and biotechnology due to its excellent properties including biodegradability, oxygen and water vapor permeability, biocompatibility and minor inflammatory response[1,2,5,11,14,19]. In our country, there are many traditional herbal that are highly effective in treating burns. Curcumin, collected from the rootstock of the perennial herb (Curcuma longa), is a yellow-orange low-molecular-weight natural polyphenol compound. Many studies have shown that curcumin has numerous good properties such as antiviral, antibacterial, antifungal, antioxidant, antiinflammatory, anticancer, anticoagulant and so on[17,18]. As a result, curcumin is applied in many biomedical products to treat wounds, burns, diabetic ulcers and stomach ulcers. In addition, many researches suggest that curcumin which conjugates with synthetic and natural polymer films can be potentially used as wound dressing[12,13,25].

At the same time, coconut oil is a natural oil available with a high percentage of unsaturated short-chain fats, capable of polymerizing with silk fibroin that stretches the silk polymer chain to become flexible, helping the membrane to increase elasticity[6]. Therefore, FCFs which were fabricated by combining silk fibroin, turmeric powder and coconut oil could be used as a promising wound dressing for wound healing and skin regeneration.

Material and Methods

Cocoons of silkworm *Bombyx mori* were provided by Vietnam Sericulture Joint Stock Corporation at Bao Loc city, Viet Nam**.** Curcumin flour and coconut oil were provided by Traditional Medicine Institute, Ho Chi Minh city, Viet Nam. All chemicals, applied in this research were supplied by Sigma Aldrich Company (USA).

Fabricating films from fibroin/curcumin combined with coconut oil (FCF): Cocoons of silkworm *Bombyx mori* were boiled for 45 minutes in a solution of 0.5% Na_2CO_3 and washed several times with distilled water to remove sericin. Then, fibroin fibers were desiccated at 50 - 60˚C in drying cabinet. Degummed silk fibroin fibers were first dissolved in 98% formic acid implement $CaCl_2$ (½ of fibroin fibers weight) with fibroin concentrations 3% (w/v) and curcumin powder for 45 minutes.

Then coconut oil was added to the final mixture with concentrations 0.4%, 0.6% (v/v) respectively. After thoroughly mixing, 25 mL of the above mixture was pipetted into a 90 mm Petri dish and dried in fume hood for 2 days to prepare the FCFs which were washed with distilled water, packed and sterilized gamma irradiation 25 kGy.

SEM analysis: The FCFs were examined by Scanning electron microscope (SEM, JSM-6510, JEOL, Japan) at Vietnam Academy of Science and Technology, Ho Chi Minh city, Viet Nam.

Tensile strength: The FCFs were examined by EZ50 Universal Testing Machine (Lloyd Instruments, UK) at Vietnam Academy of Science and Technology, Ho Chi Minh city, Viet Nam.

Water uptake: The maximum water uptake of the FCFs was evaluated. The FCFs were cut into 1cm x 1cm specimens. The weights of specimens were recorded as M1; then, specimens were immersed in 40 mL of the solution A (2.298g NaCl, 0.368g $CaCl_2.2H_2O$ of deionized water) for 45 minutes at 37±1°C. Then, the redundant surface water on the specimens was absorbed by a filter paper and the weight of FCFs was recorded as M2[7]. The equation for the water uptake capacity (W) calculation is as follows:

$$W = (M2-M1) \times 100\%/M1$$

Water vapor transmission rate (WVTR): The FCFs were cut into 1cm x 1cm specimens; then, the specimens were immersed in 40 mL of the solution A for 45 minutes at 37±1°C. Then, the redundant surface water on the specimens was absorbed by a filter paper and the weight of samples was recorded as M1. The wet specimens were put into incubator for 24 hours at 37±1°C; then the specimens were reweighed as M2[3]. In order to calculate the water vapor transmission rate (WVTR), the following equation was used:

$$WVTR\ (g/hours) = M1-M2/T$$

where T was 24 hours.

Degradation: The FCFs (1cm x 1cm) prepared were weighed before simulating the degradation testing. The samples were placed in 10 ml solution A at 37±1°C. The samples were taken out; then, the redundant surface water on the specimens were absorbed by a filter paper. Finally, the samples was re-weighed at different time points (6, 12, 18, 24, 30 ,36, 42 and 48 hours)[7].

pH changing test: The FCFs (1cm x 1cm) prepared were immersed into distilled water (pH=7) at 1:100 (w/v) for 24 hours at room temperature. The pH of solution was evaluated by pH meter after 3 hours and 24 hours[7].

Bacterial penetration test: The FCFs were cut into circular discs with 15 mm diameter and covered on the agar plates. Later, the agar plates were placed in the work environment for 24 hours.

The agar plates not covered by FCFs were in the control group. Then the agar plates were placed at 37°C in an incubator for 20 - 24 hours. The presence of bacteria was observed after the samples were removed[3].

***In vitro* cytotoxicity:** The cytotoxicity of FCFs was determined by the direct contact with human fibroblasts (hFs) according to the guideline of ISO 10993-5, in which a latex material was used as a positive control causing toxicity for hFs.

Initially, hFs were seeded in a 35 mm culture dish at 10^5 cells/dish and cultured for 24 hours for cell attachment. When cells confluence about 95%, all samples were cut into specimens of 1 × 1 cm^2 and placed on the tested dish. After incubation at 37°C for 24 hours, tested samples were removed; then, the hFs were examined microscopically for cellular morphology and response around the samples.

Statistics analysis: GraphPad Prism 7.0 was used to conduct the statistical analysis (GraphPad Software Inc., San Diego, CA, USA). P 0.05 was considered statistically significant. ImageJ version 1.52e was used to create the images (National Institutes of Health, Bethesda, Maryland, U.S.).

Results

Fabricating FCFs: The initial silk had a rough sericin layer covering fibroin fiber inner (Fig. 1A). After degumming process, sleek fibroin fibers (Fig. 1B) were received; it suggested that the sericin layer of silk was completely removed. After the formic acid completely evaporated, the remaining elements would restructure quickly and create a film with a similar morphology (Fig. 2).

Surface morphology of FCFs: The FCFs with 0.4% and 0.6% coconut oil concentration had a cross-woven structure that created holes on the surface (about 40µm in diameter) (Fig. 3).

Mechanical tensile of FCFs: FCFs had mechanical tensile decreasing by coconut oil concentration from 10.57 MPa to 6.82 Mpa (Fig. 4).

Water Content Measurements: All FCFs had very high absorbency (about 154%). Statistically, the absorbency rates of FCFs formed with different coconut oil concentrations were the same (Fig. 5).

Water vapor transmission rate (WVTR): All FCFs had ability of dehydration. Statistically, the WVTRs of FCFs formed with different coconut oil concentrations were the same. Therefore, FCFs could provide wound areas with suitable moist environment (Fig. 6).

Degradation properties of FCFs: All FCFs with different coconut oil concentrations had unchanged weight after 48 hours (Fig. 7).

pH changing test: The pH change of FCFs after 3 hours was within range of 6.44 - 6.55; after 24 hours the pH change of samples increased from 6.72 to 6.76. Statistically, the pH change of FCFs differed between 3 hours and 24 hours (Fig. 8).

Bacterial penetration test: The results (Fig. 9) showed that the agar surface of plates was covered by FCFs with different coconut oil concentrations and showing no bacterial colonies growth; however, the control plates presented bacterial colonies and fungal spores, which indicated that the FCFs is an effective bacterial barrier.

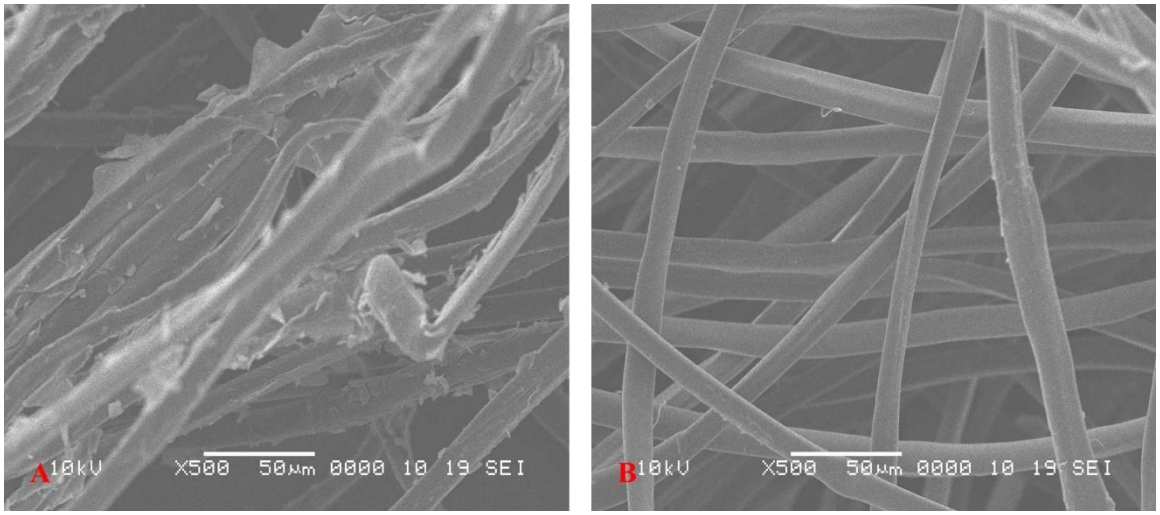

Figure 1: SEM analysis of silk fibers (X500 magnification). (A) Silk before degumming. (B) Silk after degumming.

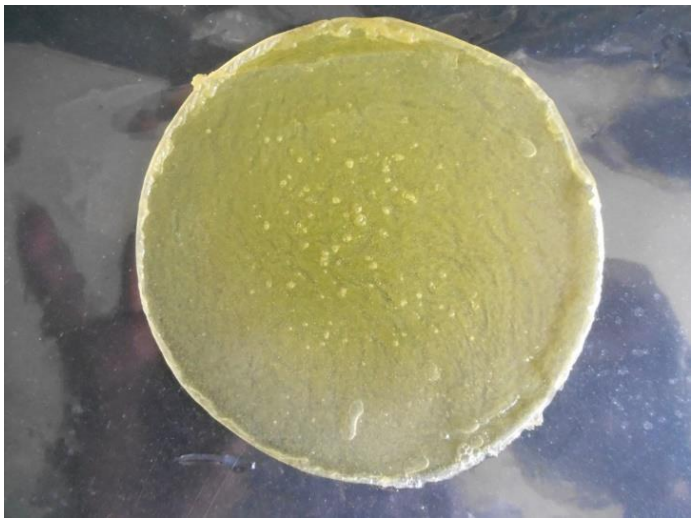

Figure 2: Morphology of FCFs.

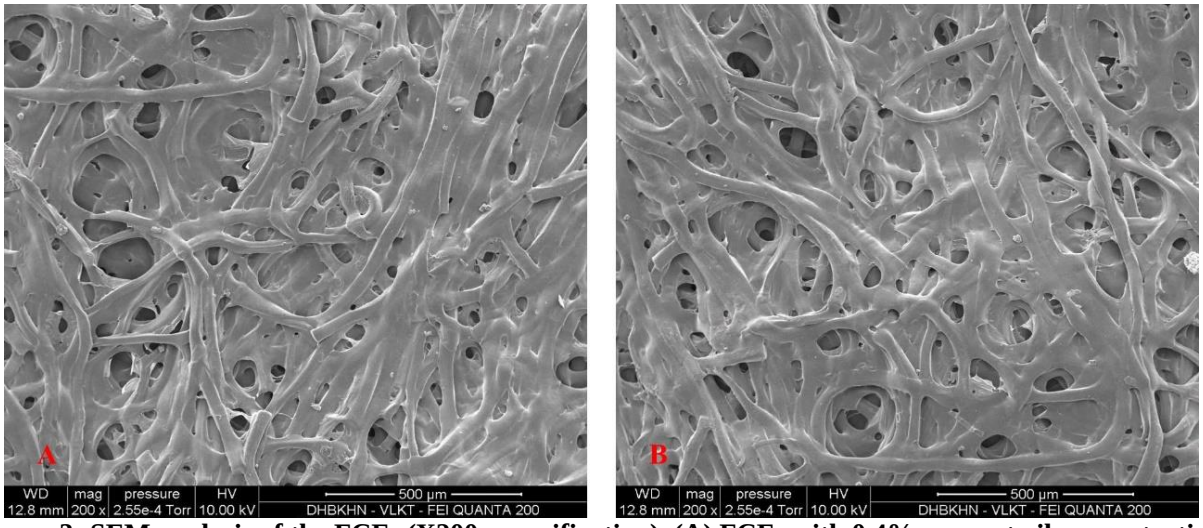

Figure 3: SEM analysis of the FCFs (X200 magnification). (A) FCFs with 0.4% coconut oil concentration. (B) FCFs with 0.6% coconut oil concentration.

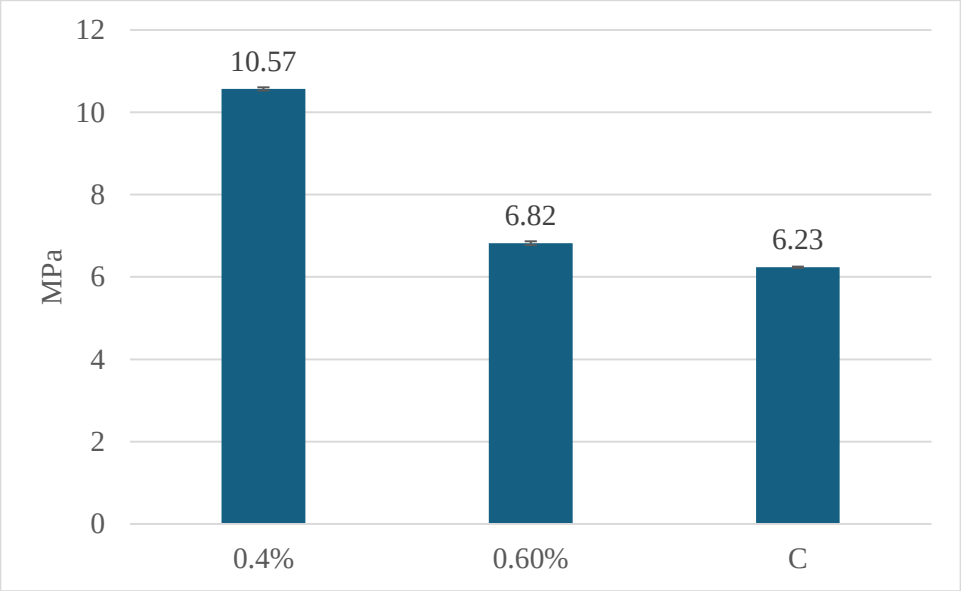

Figure 4: FCFs mechanical properties evaluation (C- trading wound dressing).

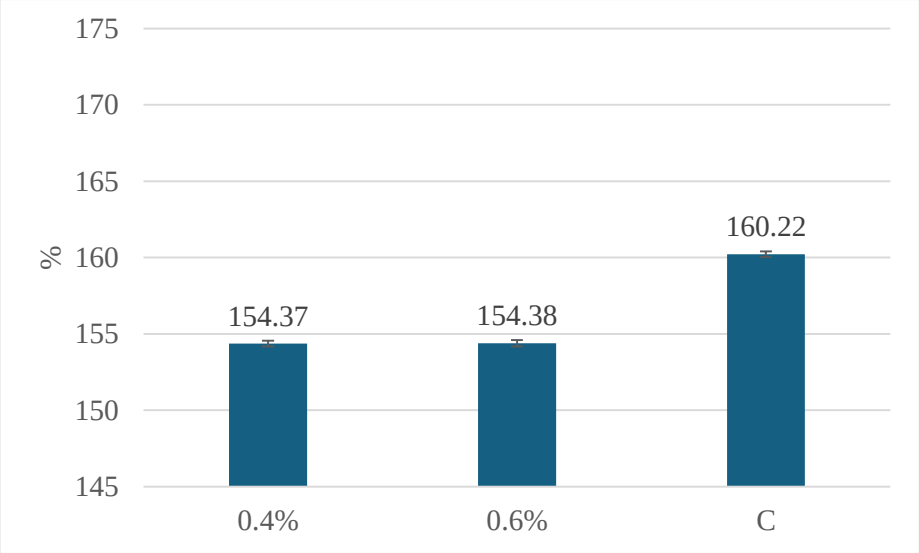

Figure 5: The water uptake of FCFs.

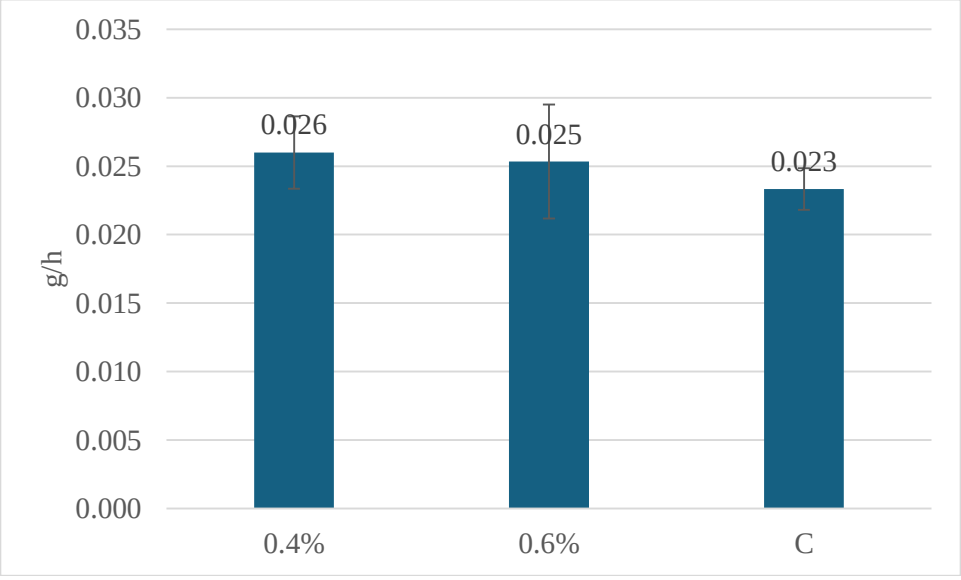

Figure 6: Water Vapor Transmission Rate of FCFs.

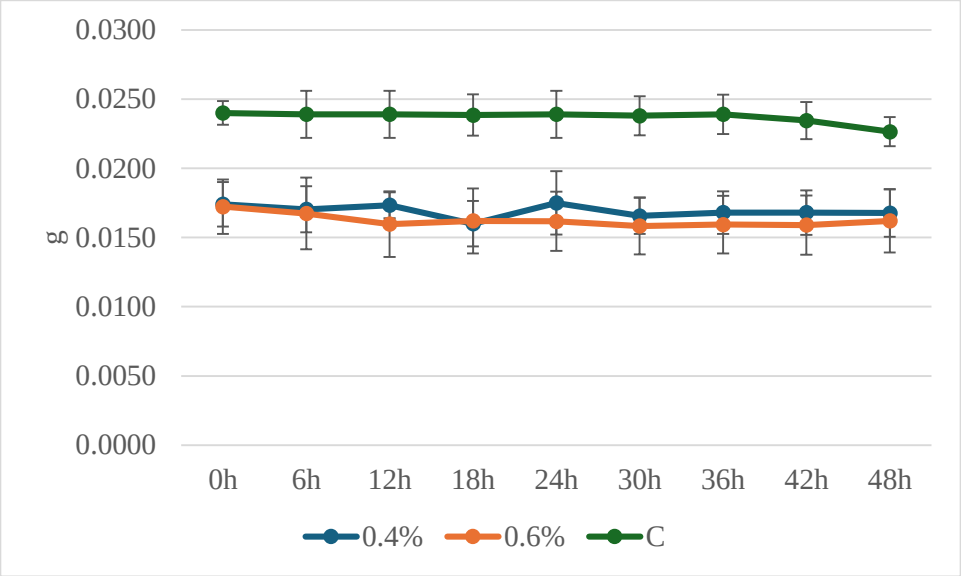

Figure 7: Weight loss of FCFs at different degradation time points.

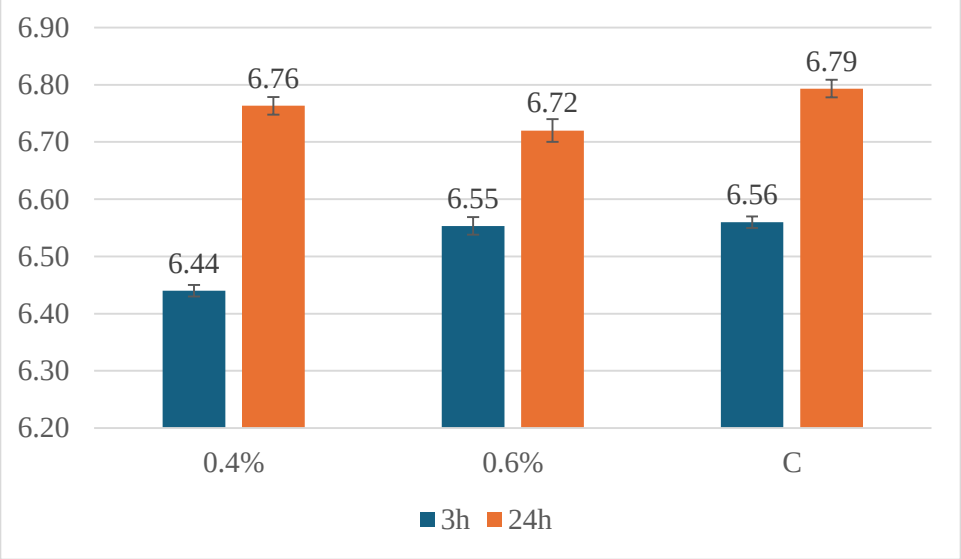

Figure 8: pH change of FCFs after 3 hours and 24 hours.

Cytotoxicity test: The cells surrounding and below the FCFs had normal morphology (Fig. 10 C, D). The cells in the control dish were mostly rounded, floating on the surface or were deformed (Fig. 10A). According to the ISO 10993-5 guidelines, all FCFs with different coconut oil concentrations had cytotoxic levels at 0. This result revealed that FCFs exhibited good biocompatibility.

Discussion

The original silk included two parallel fibroin fibers which were covered by the layer of sericin on their surface. However, sericin was the crucial cause of non biocompatibility and allergies of silk. Silk degumming was the process of demolishing the peptide bonds between sericin and fibroin that helped to remove the sericin layer. Many previous studies demonstrated that alkaline solution at high temperature was an effective and appropriate degumming method[20]. In general, aqueous SF solution was obtained by dissolving SF in the concentrated neutral salts such as $CaCl_2$, LiBr and so on[24].

However, SF solution became unstable and precipitated because of low solubility in water when storing for long time. As a result, it was necessary to find out a good solution system having high stability and easy processibility. Formic acid was known as a good solvent because this solvent allowed producing a stable solution and had an excellent film and fiber formation properties for silk protein polymer. The formic acid solution had many ions which would link to bonds of fibroin fibers that helped to dissolve and prevent these fibers from binding together[23].

However, when formic acid evaporated completely, the closer fibroin fibers would re-form hydrogen bonds making the chains less flexible and the resulting film became stiff and brittle.

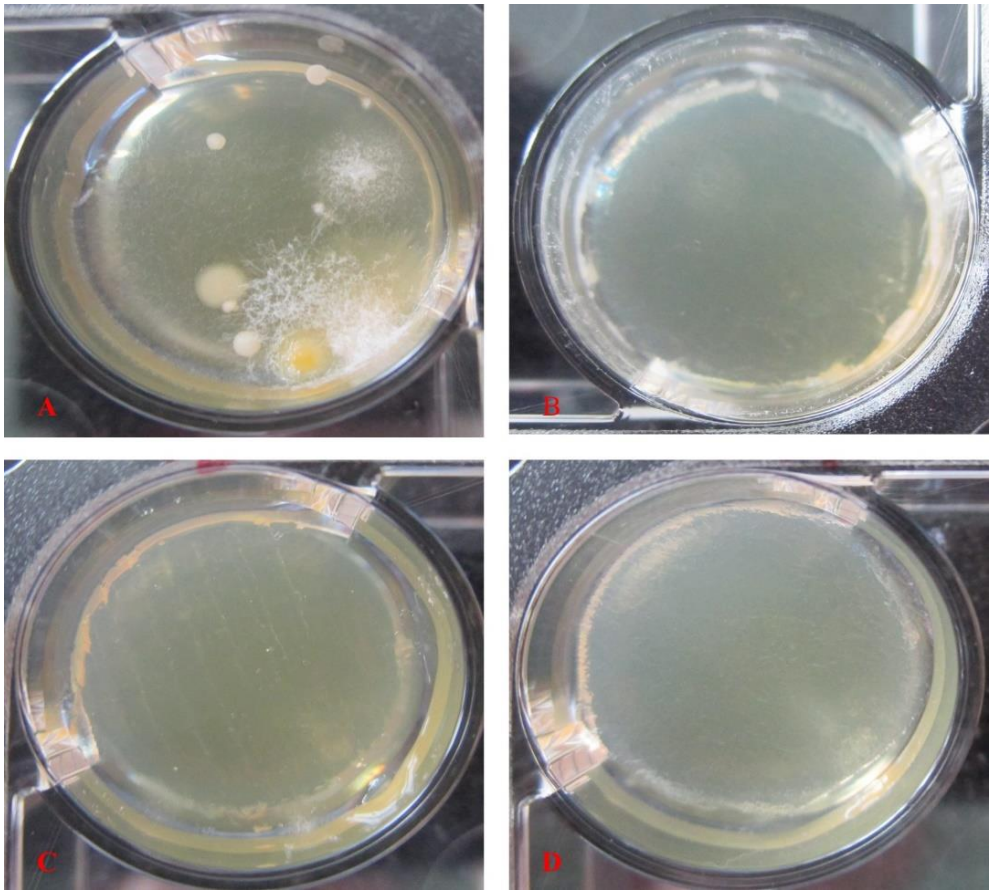

Figure 9: Prevention of FCFs to penetration of bacteria. (A) Control. (B) Commercial wound dressing. (C) FCFs with 0.4% coconut oil concentration. (D) FCFs with 0.6% coconut oil concentration.

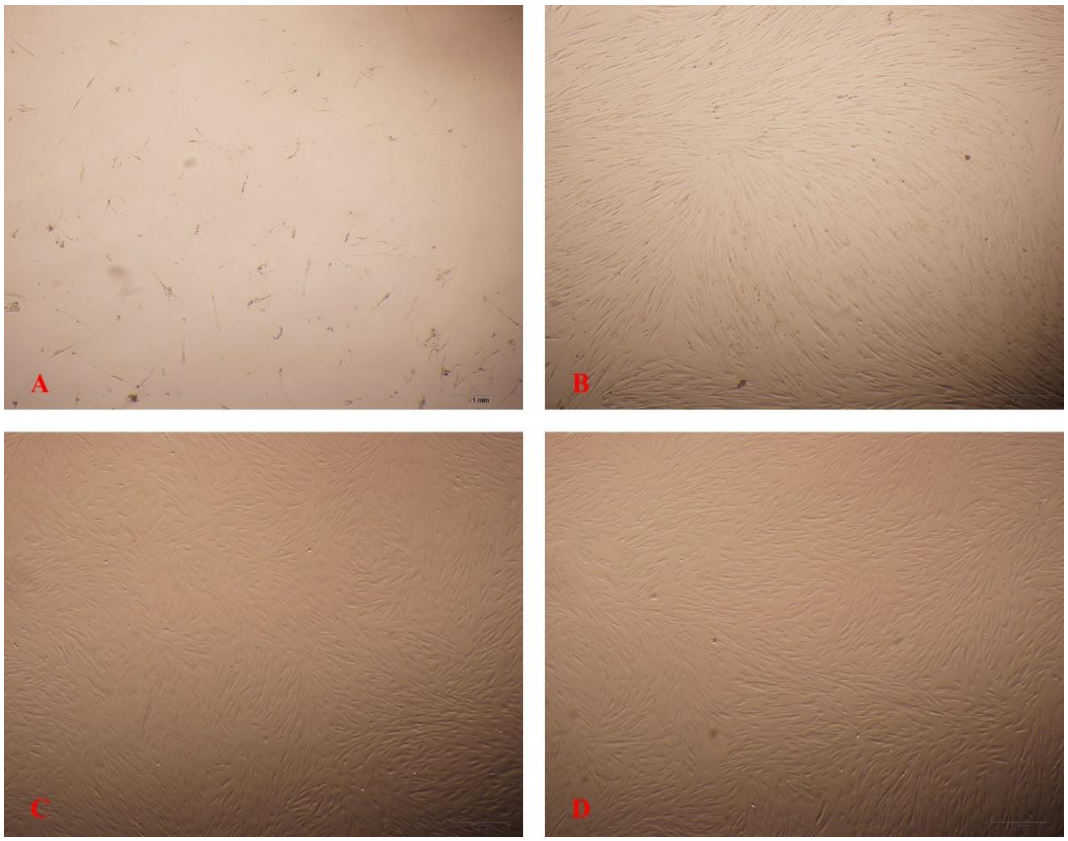

Figure 10: Direct contact test. (A) Control (latex). (B) Commercial wound dressing. (C) FCFs with 0.4% coconut oil concentration. (D) FCFs with 0.6% coconut oil concentration.

Therefore, coconut oil was added because the oil contained short and medium chain fatty acids. The -COOH group and carbon chains of these fatty acids would react with the -NH group in fibroin (polymer condensation reaction). Immediately after the reaction, the carbon chains of these fatty acids would penetrate the fibroin chains, acted as a plasticizer, pushed the fibroin chains away and made it impossible for hydrogen bonds to re-establish (even when formic acid evaporates completely)[6]. The resulting film would be more flexible and resilient.

As previous studies of solution stability and crystallization of SF molecules in formic acid, formic acid induced the β-sheet crystallization of SF during drying process by eliminating the formic acid from inside SF polymers[8]. The crystallization induced by formic acid favors the formation of short-range ordered structure, thereby creating FCFs with a cross-woven structure that created holes on the surface (about 40µm in diameter). The results of SEM image showed that FCFs with different concentrations of coconut oil had similar surface structures, indicating that coconut oil did not affect the film-forming ability and the film structure.

The restructuring of β-sheet gave FCFs high mechanical properties. Besides, the mechanical properties were inversely proportional to the coconut oil concentration, indicating that the addition of coconut oil increased the flexibility but reduced the mechanical properties of FCFs. However, FCFs with different coconut oil concentrations all met mechanical property standards. The commercial wound dressing had mechanical tensile similar to human normal skin (≥1.8 MPa)[5,9].

Generally, bioactive substances could be bundled onto fibrous biomaterial films by adsorption. Since many bioactive substances had suitable affinity, silk fibroin had potential for drug delivery system applications. Curcumin was hydrophobic mainly bound with the hydrophobic domains of SF in FCFs through non-covalent bonds occurring at the amide group regions or β-sheet regions. In addition, curcumin was adsorbed hardly at the surface of FCFs, but could interact with the core vesicles of fibroin[10].

The releasing curcumin from hydrophobic domains of SF in FCFs mainly depended on the features of inner hydrophobic domains and the linking between bioactive substances and this domains. The sustained releasing curcumin from the FCFs depended on non-covalent interactions between curcumin and fibroin. In addition, many researches showed that curcumin could release from curcumin-containing films through diffusion mechanisms[12].

Most wound dressings caused the wound pH to increase after 24 - 26 hours. During process of wound healing, the wound pH changed from neutral to acidic. The wounds which had pH ≤ 7.6 had wound closure increased by 30% after 2 weeks treatment[21]. The changing pH of FCFs after 3 hours and 24 hours were suitable to promote wound healing. In current wound care processing, if the wound dressings could not effectively protect against the invasion of external bacteria and fungus, it was easy to cause secondary bacterial infection[4]. The bacterial barrier properties of FCFs could be due to their linked fibrous structure that prevented the penetration of infectious agents. In addition, the antibacterial properties of curcumin also supported this property.

Conclusion

In this study, FCFs were successfully fabricated with silk fibroin as the matrix and curcumin flour as the bioactive element. At the same time, coconut oil was added to increase flexibility as well as to adjust the mechanical properties of FCFs to suit different clinical conditions. The obtained results suggested that the FCFs had suitable rate of water uptake (154%) and conformable tensile strength (≥1.8 MPa). Surface with many holes could supply a conformable moist environment for wounds, effectively protected against bacterial infection. Results of cytotoxicity test showed that the FCFs were having good biocompatibility with hFs. Therefore, the FCFs with 0.4% and 0.6% coconut oil concentrations were a good choice for wound dressing applications.

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