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Effects of crosslinking agent and biological properties of silk fibroin/gelatin/ chitosan ternary system electrospun nanofiber mats

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Abstract

In this study, electrospinning technique was used for ternary system to fabricate nanofiber mats from silk fibroin (SF):gelatin (G):chitosan (C) with various mass ratios i.e. 10:20:0, 10:20:0.5, 10:20:1, 10:20:1.5, 10:20:2, and 20:10:1. An increase in chitosan content of the mats was found to decrease average fiber diameter and with narrow size distribution. Tensile strength of SF:G:C nanofiber having greater SF content was lower than that of the fiber mat having lower SF content. The obtained fiber mats were then crosslinked by three different crosslinking agents including ethanol, glutaraldehyde and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC)/N-hydroxysuccinmide (NHS). The smooth fiber with high porosity of the nanofiber mat was observed after crosslinked using EDC/NHS agent. A less swollen fiber was noticed in the fiber mats having higher SF content. The results from in vitro study revealed the good cell adhesion and proliferation of gingival tissues. Such results indicated the potential use of SF:G:C nanofiber mats as membrane application.

1. Introduction

Guided bone regeneration (GBR) is an important therapy to repair mandible and alveolar bone defects affected by periodontal diseases. In this technique, a barrier membrane is adapted to help prevention of ridge resorption after extraction, augmentation of alveolar ridge defects and improvement of bone healing around dental implants [1]. The barrier membranes are categorized into two types based on their resorbability i.e. non-resorbable and resorbable membranes. Commercial non-resorbable membranes are made from synthetic polymer such as expanded-polytetrafluoroethylene (ePTFE: Gore-Tex®), cellulose acetate (Millipore filter), and polytetrafluoroethylene (PTFE: TefGen-FD®) whereas resorbable membranes are made from either synthetic polymer such as polylactic acid (Guidor®), polylactic/polyglycolic acid (Vocryl®) or natural materials such as collagen (Bio-Gide®, BioMend®) [2].

Materials for membrane fabrications have been extensively developed over the years in clinical field as the utilization of membrane based techniques tends to increase. The materials must stay intact as physical barriers with the ability to take out unwanted cells until regeneration is complete, yet not interfere with the growth of newly formed tissue. Each material has its advantage and disadvantage inherent for the application in which it is insuring success. The biological and physical characteristics of biomaterials used to manufacture membranes can significantly influence barrier function as well as host tissue reaction.

Physical characters of the barrier membrane including pore size, tri-dimensional topography and method of membrane fabrication play an important role in GBR. The pore size of the barrier membrane affects the prevention of excessive fibrous tissue penetration into the bone defect therefore allow neovascularization and bone formation [3]. Pores membrane are necessary for cell migration [4]. It can change the cell occlusion properties and the biological reaction of different cell types to the membrane.

Electrospinning is a novel membrane fabrication method which can produce ultrafine fiber in a level of microns to nanometers and produce the tri- dimensional structure [5]. Silk, gelatin (G), and chitosan (C) are bunch of natural materials in Thailand that can be constructed and utilized in medical treatments due to inexpensive, biocompatible and biodegradable. Moreover, those kinds of materials can be fabricated into membrane by electrospinning technique [6-8].

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Nang noi Srisaket 1, silk fiber from one of silk races, is a natural and an economical fiber in Thailand. Structurally, each silk consists of two main proteins, silk sericin and silk fibroin (SF). SF possesses potential properties as a resource of biomaterials because it is non-toxic, good biocompatible and biodegradable [9-10]. It has been widely used in medical application for sutures, skin tissue and artificial ligaments [9-11]. Gelatin (G) is a natural polymer, which can be generally found in animal tissues such as skin, muscle, and bone. Because it has biocompatibility and biodegradability, gelatin has been commonly used in biomedical applications [12]. In addition, gelatin is a promising choice to be used for nanofiber production as it is cheap and available [13]. Chitosan (C) can be prepared from chitin mostly found in crustacean shell. It is ranked number two being found in natural materials. Chitosan has a unique property of antimicrobial activity that is useful in medical fields. It is biocompatible and biodegradable. Furthermore, it can be applied for wound healing [14], periodontal treatment [15], and tissue engineering [16]. Polymer blends combine outstanding properties of each polymer

Currently, the preparation of biopolymer blends and their applications have been widely studied for the improvement of the biomaterial properties such as collagen/chitosan blends [17], chitosan/silk fibroin blends [9,18,19], silk fibroin/gelatin blends [20]. However, the effective solvent to dissolve such copolymers is the major problem. Formic acid [21-22] is extensively used as solvent of silk fibroin (SF), gelatin and chitosan for electrospinning fabrication. Therefore, the dissolution of the SF:G:C ternary system in formic acid was studied.

The as-spun fiber mats of natural polymer exhibit unstable, poor mechanical properties and water soluble. Therefore, crosslinking process is required to chemically join two or more molecules by a covalent bonding. The crosslinking agents for such biopolymer normally included glutaraldehyde (GA) [23], ethyl alcohol (EtOH), 1-ethyl-3-(3-dimethylaminopropyl) cabodiimide (EDC)/ N-hydroxysuccinimide (NHS) [24].

This research aims to prepare fiber mats from SF:G:C via electrospinning technique. The effects of various polymer ratios and crosslinking agents on physical, chemical and biological properties of the SF:G:C fiber mats were investigated. The biological properties of the obtained fiber mats were also benchmarked to those of commercial membranes.

2. Materials and methods

2.1 Materials

The experiments were conducted using silk, gelatin and chitosan. Silk (bombyx mori cocoon, Nang Noi Srisaket1) was provided by the Queen Sirikit Department of Sericulture, Ministry Agriculture and Cooperatives. Chitosan having molecular weight of 300,000 Da and 75-80% degree of deacetylation was obtained from Biolife Co., Ltd., Thailand. Gelatin from porcine skin was purchased from Fluka-Aldrich, Germany. The chemicals used for the preparation of silk fibroin were sodium carbonate (Na_2CO_3), calcium chloride (Ca_2Cl) and ethanol (CH_3CH_2OH) from Merck, Germany. Formic acid for spinning solution having concentration of 90% was obtained from Merck, Germany. All solutions used in this study was applied without any further purification.

2.2 Preparation of SF:G:C solution

Regenerated silk fibroin sponge was prepared by the following procedure. Fresh silk cocoons were degummed with 0.02M Na_2CO_3 at 100°C for 1 h and subsequently rinsed with distilled water. The process was repeated twice to remove the glue-like sericin proteins. The obtained fibroin proteins were then dried at room temperature. The degummed silk fibroin was dissolved in co-solvent of Ca_2Cl/ethanol/water at molar ratio of 1:2:8 at 80°C for 6 h. The solution mixture was dialyzed using tubular cellulose membrane (molecular weight cut off 12,000) in deionized water for 4 days at room temperature. The fibroin solution was then lyophilized to obtain silk fibroin sponge.

The SF:G:C solution was prepared by dissolving 1wt% of chitosan powder in 90% formic acid until the homogeneous solution was obtained and further mixed with silk fibroin sponge and gelatin at various ratio. The mixture was continuously stirred at a room temperature for 1 h.

2.3 Electrospinning of SF:G:C solution

The obtained solution was filled in 5 ml syringe equipped with a stainless steel needle and then attached to a syringe pump with a controlled volume flow rate of 0.2 ml/h. The tip of syringe needle was connected to a power supply. A rolling stainless drum was used as a fiber collector. Electric potential was controlled at 10-20 kV at distance from the syringe tip to the collector was in a range of 10 to 20 cm. The nanofiber mats on a collector were dried overnight in desiccator to remove residual solvent and moisture before measurements. The experimental set up for electrospinning of the SF:G:C solution was illustrated in Figure 1.

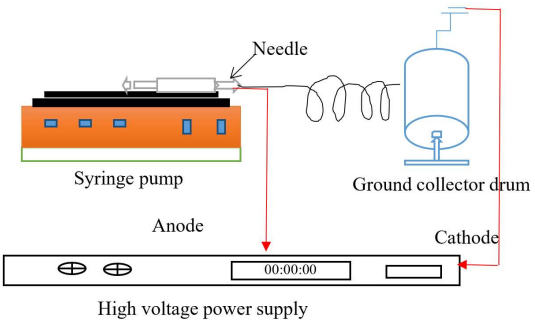


Figure 1. Electrospinning process for fabrication of SF:G:C fiber mats.

2.4 Crosslink of nanofiber mats

The dry electrospun nanofiber mats were then crosslinked using the method of chemical vaporization with and soaking in three different crosslinking agents including ethanol (EtOH), glutaraldehyde (GA), and 1-ethyl-3-(3-dimethylaminopropyl) cabodiimide (EDC)/ *N*-hydroxysuccinimide (NHS). The fiber mats were cut into small pieces of 5×5 cm^2 and subjected to 95% crosslinking agent vapor for 72 h and further soaked in the crosslink solution for 10 min then washed with pH 7.4 PBS solution to remove the residual crosslinking agent. Lastly, the nanofiber mats were dried in a desiccator. The same procedure to crosslink fiber mats was applied for all three crosslink agents. The 2:1 weight ratio of EDC:NHS in 95% EtOH was prepared as EDC/NHS crosslinking agent. The chemical vaporization was introduced in order to pre-crosslink gelatin and reduce the solubility of the nanofiber mats when soaking in crosslink solution [25].

2.5 Characterization of the nanofiber membranes

2.5.1 Physical and chemical characterization

The morphology of nanofiber mats before and after crosslinked was evaluated using scanning electron microscope (SEM) JEOL, JSM-6400. The dry nanofiber mats were cut into small pieces and fixed on the stubs then coated with gold particles. The average diameter of electrospun nanofiber mats was determined by measuring the diameter of the nanofiber in the SEM images by Image J program version 1.48. The fiber diameters were presented as the average standard deviation.

The tensile properties of the as-spun fiber mats were investigated according to DIN 53504-S2 standard. The samples having 75 mm full length, 25 mm parallel length, 20 mm gauge length and 12.5 mm width were fabricated. The test was performed using a crosshead speed of 1 $mm \cdot min^{-1}$ under an ambient condition. The reported data were average from five repeated measurements.

The efficiency of each crosslinking agent was evaluated by weight loss and degree of swelling of the fiber mats, calculated as equations 1 and 2, respectively. The weight loss (%) and the degree of swelling (%) of each sample were calculated according to equation 1 and 2, respectively.

$$\text{Weight loss (\%)} = \frac{(W_b - W_d)\times 100}{W_d} \quad (1)$$

$$\text{Degree of swelling (\%)} = \frac{(W_s - W_d)\times 100}{W_d} \quad (2)$$

W_b represented the initial weight of the crosslinked nanofiber mats (g), W_s was the weight after submerged in distilled water for 2 h and removed the excess water (g) and (W_d) was the weight of the samples after dried in a desiccator for 72 h.

2.5.2 Biological characterization

The non-crosslinked and crosslinked nanofiber mats with a dimension of 0.7×0.5 cm^2 were soaked in PBS for 10 min, then immersed in Dulbecco's Modified Eagle Medium (DMEM) with 5% antibiotics-antimycotics solution for 1 h and washed with DMEM twice before testing.

Normal human gingival fibroblasts (HGF) were prepared from healthy donated gingival tissues. The tissue samples from gingivectomy were cut into small pieces and transferred to 35 mm culture plates (Falcon, Germany). The tissue samples were cultivated in DMEM supplemented with 10% fetal calf serum, 1% L-glutamine, 100 units/ml penicillin and 1% antibiotic antimycotic solution (Gibco BRL, USA) and maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The fresh medium was daily replaced. The explants were observed under the inverted microscope. The subculture of the cell clusters ware performed in order to reduce the density of cell population and evenly distributed the cells in the new culture plates.

The cell suspension was filtered with lens paper, and then centrifuged at 2,000 rpm. The supernatant was discarded. The pellet was re-suspended in the fresh medium. The cells having the density of 4×10^4 cells per ml were incubated in culture medium and kept at a temperature of 37°C in a humidified atmosphere of 95% air, 5% CO_2. Cells from the fifth passage were used to study the fibroblasts response to the membrane.

The morphology of HGF after biological test was observed using SEM technique. The membranes were washed thrice with 0.1 M PBS at pH 7.4 and fixed with 2% glutaraldehyde in 0.1 M PBS with a controlled pH 7.2 and a temperature of 4°C for 1 h and 1% osmium tetroxide for 1 h. The specimens were dehydrated with graded ethanol each condition 35%, 50%, 75%, 95%, and 100% of ratio, respectively for 15 min. The specimens were then dried at a critical point drying point with liquid nitrogen, fixed on the stubs and coated with gold particles before investigation.

3. Results and discussion

3.1 Effect of polymer ratio on morphology of nanofibers

Polymer ratio is a key factor to control the diameter of nanofiber produced from electrospinning. The SF:G:C blended solutions at ratios of 20:0:0, 0:24:0, 10:20:0, 10:20:0.5, 10:20:1, 10:20:1.5, 10:20:2, and 20:10:1 were spun at an electrical field of 10 kV and a spinning distance of 10 cm at feeding rate of 0.2 $ml \cdot h^{-1}$. The morphology and fiber diameters of as-spun SF: G: C blends were displayed in Figures 2(a) and 2(b).

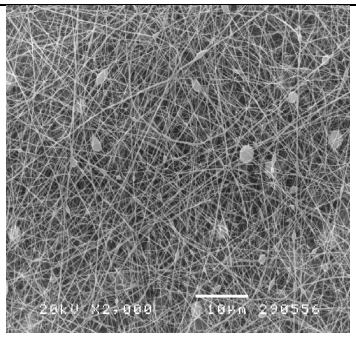
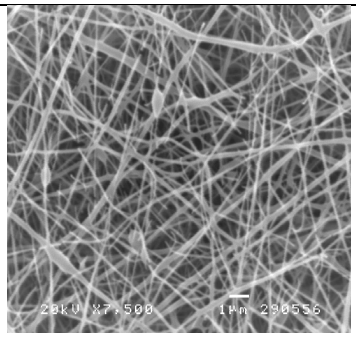
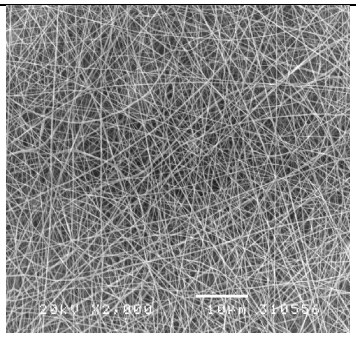
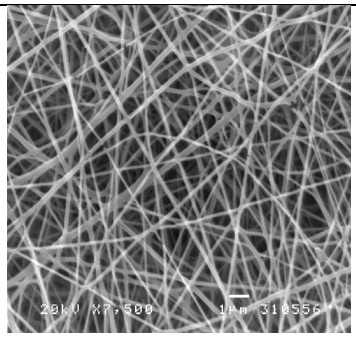
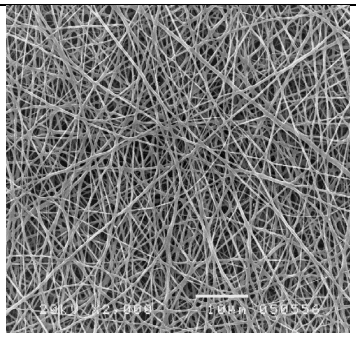
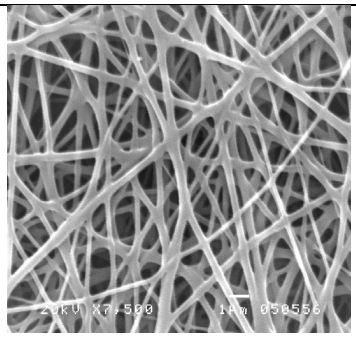
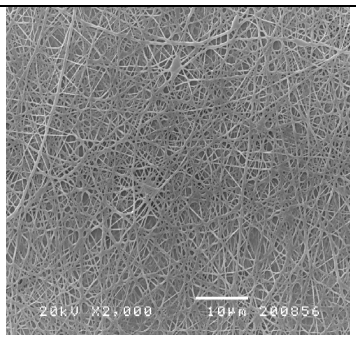
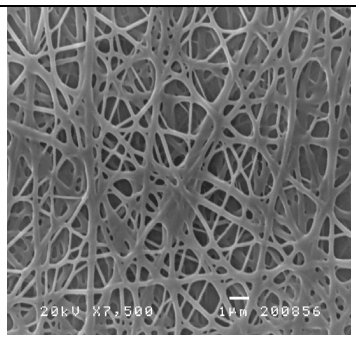
SF:G:C weight ratio	Magnification		Fiber diameter
	2,000x	7,500x	
20:0:0			109 ± 23 nm
0:20:0			148 ± 48 nm
10:20:0			286 ± 18 nm
10:20:0.5			202 ± 77 nm

Figure 2a. SEM images of electrospun nanofiber SF:G:C at various concentration of 20:0:0, 0:24:0, 10:20:0 and 10:20:0.5 produced by an electric field of 10 kV, a spinning distance of 10 cm and a feeding rate of 0.2 ml h^{-1}.

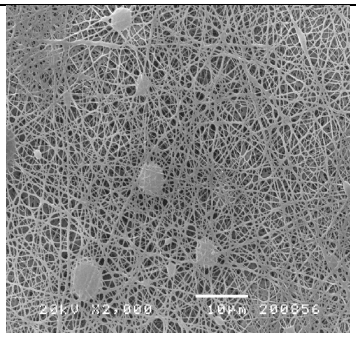
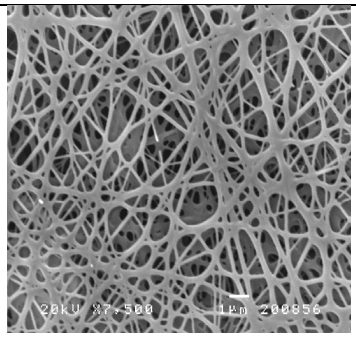
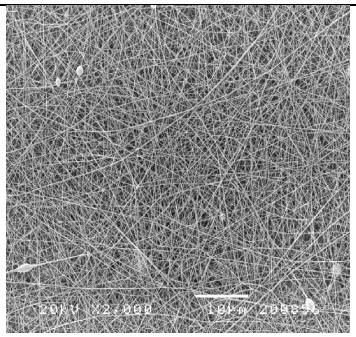
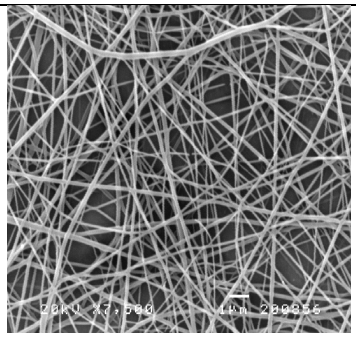
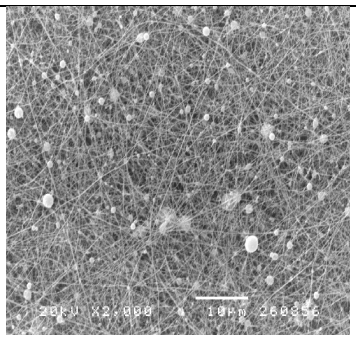
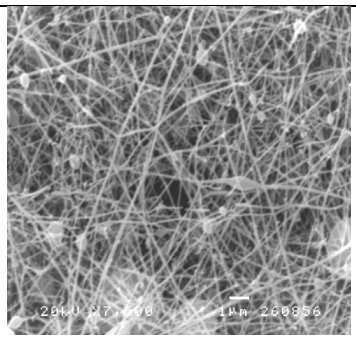
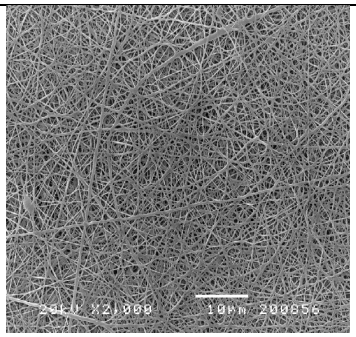
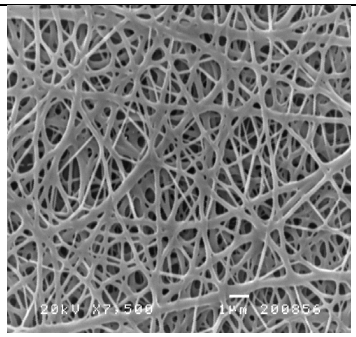
SF:G:C weight ratio	Magnification		Fiber diameter
	2,000x	7,500x	
10:20:1			223 ± 84 nm
10:20:1.5			245 ± 102 nm
10:20:2			102 ± 76 nm
20:10:1			208 ± 81nm

Figure 2b. SEM images of electrospun nanofiber SF:G:C at various concentration of 10:20:1, 10:20:1.5, 10:20:2 and 20:10:1 produced by an electric field of 10 kV, a spinning distance of 10 cm and a feeding rate of 0.2 ml h^{-1}.

It was found that the pure silk fibroin solution could be spun into fiber with some formation of bead where the pure gelatin solution produced the continuous fiber as clearly seen in Figures 2a and 2b. On the other hand, the pure chitosan could not be fabricate into fiber because of the three dimensional networks of strong hydrogen bonds in the structure of chitosan. The blend solution of SF:G:C at a ratio of 10:20:0 was prepared into nanofiber with an average diameter of 286 ± 18 nm. The result was in agreement with Okhawilai et al in 2010 [25]. They reported that the SF:G electrospun fiber was able to fabricate at the ratio of 30:70 and 40:60 having average diameter of 258 ± 60 and 304 ± 65 nm, respectively with an electrical field of 15 KV and a spinning distance of 20 cm. The results indicated that the blend of silk fibroin and gelatin in formic acid could be easily fabricate into nanofiber by electrospinning technique. With an addition of chitosan in SF:G:C fiber mats, the fiber mats exhibited smooth and continuous fiber and average fiber diameter was found to increase with increasing chitosan content. The values were 202 ± 77, 223 ± 84 and 245 ± 102 nm with SF:G:C ratios of 10:20:0.5, 10:20:1 and 10:20:1.5, respectively where the SF:G:C ratio of 10:20:2 showed the fiber mats with beads. However, with 0.5 and 1wt% of chitosan solution, the fibers fused together which might be because the electrical field and the distance were not appropriate for spinning condition. In addition, the distributions of nanofiber diameter became narrow with chitosan content. It is because an ionizable amino group in chitosan resulted in the conductivity of the electrospun blended solution [27]. The aqueous solution of formic acid provided an electric charge, thus giving repulsion in SF:G:C blended solution during electrospinning. It is possible that a single jet of polymer blend solution might produce multiple filaments during charge repulsion. It appears that an increase in the amount of chitosan gives rise to the formation of short fiber and a reduction of fiber diameter [28].

3.2 Mechanical properties of the SF:G:C electrospun fiber mats

The SF:G:C at 10:20:1 and 20:10:1 was mechanically tested by tension mode followed DIN 53504-S2 standard. The average tensile strength and elongation at break of the SF:G:C fiber mats were reported. Tensile strength of the pure G was higher than that of the pure SF as the values of the pure SF was 4.96 ± 1.46 MPa where that of the pure G was 8.60 ± 3.83 MPa. Moreover, the elongation at break of the pure G was greater than that of the pure SF as well as the values were 11.46 ± 2.08% and 9.46 ± 4.54%, respectively. No significant difference in tensile strength of the SF:G:C nanofiber was observed as the values were 5.48 ± 0.72 and 4.04 ± 1.08 MPa in the 10:20:1 and 20:10:1 SF:G:C fiber mats, respectively. Whereas, the elongation at break of the SF:G:C having greater G content was higher than the other. The value of 10:20:1 SF:G:C fiber mats was 13.51 ± 2.22% where that of 20:10:1 SF:G:C was 6.53 ± 2.19%. It might be due to a greater fiber diameter of the SF:G:C at 10:20:1 resulting in a greater elongation of fiber before breakage.

3.3 Effects of crosslinking agents on physical properties of the SF:G:C electrospun fiber mats

To eliminate beads and prevent fused fiber, the processing condition for electrospinning was adjusted to be 20 kV of applied voltage and 15 cm of distance from the tip of needle to collector. Figure 3 shows SEM micrograph of the obtained nanofiber mats revealing the uniform, smooth and bead free nanofibers in the fiber mats of SF:G:C at ratios of 10:20:1 and 20:10:1. The as-spun nanofiber mats with various SF:G:C ratios were crosslinked by three crosslinking agents including EtOH, GA and EDC/NHS before further characterization. Figure 4 shows %weight loss and %swelling of the fiber mats after crosslinked.

The pure SF fiber mats after EtOH crosslinked showed about 10% of swelling where no weight loss was observed. On the other hand, the pure G fiber mats lost almost 60% of their weights after crosslinked by EtOH. The SF:G:C fiber mats at 20:10:1 after EtOH crosslinked was observed to exhibit lower weight loss than that of 10:20:1 which was attributed to that SF fiber mats showed lower weight loss compared to G fiber mats. The %weight loss of SF:G:C fiber mats crosslinked by EtOH having ratios of 10:20:1 and 20:10:1 was 8 and 1%, respectively. The results indicated the potential use of EtOH crosslinking agent to crosslink and prevent water-soluble gelatin loss. The EtOH vapor allowed partially crosslinking gelatin molecules and then the EtOH dipping further helped crosslinking the residual gelatin molecules. Moreover, there was no report on weight loss and morphology change of the β- sheet conformation of silk fibroin after rinsing. In this research, none of silk fibroin nanofiber mats lost during rinsing. It was contributed to the transformation of the random coil conformation of silk fibroin to β-sheet after EtOH crosslinked [29,30]. However, by EtOH crosslinking agent, the fiber mats.

The highest weight loss of the fiber mats was observed using GA crosslinking agent. The values were 10% equally for both 10:20:1 and 20:10:1 SF:G:C ratios. Moreover, the much shrinkage in all direction and color change of the fiber mats was noticed. It was because aldehyde group (-CHO) on GA could react with amino groups of lysine residues of protein to form the –C≡N group. The amino groups on gelatin were substituted totally by the quaternary ammonium salt groups and the residual amino groups might react with GA and formed the –C≡N- group, a chromophore. Therefore, the color of crosslinked blend mats was discriminated by a slow change in color from white to yellow. The color change occurred because the cross-linkage (CH≡N) reaction took place during the cross-linking process [31,32]. The swollen morphology of the GA crosslinked fiber mats were observed. The results

were in consistence with the greater %swelling value than the other crosslinking agents.

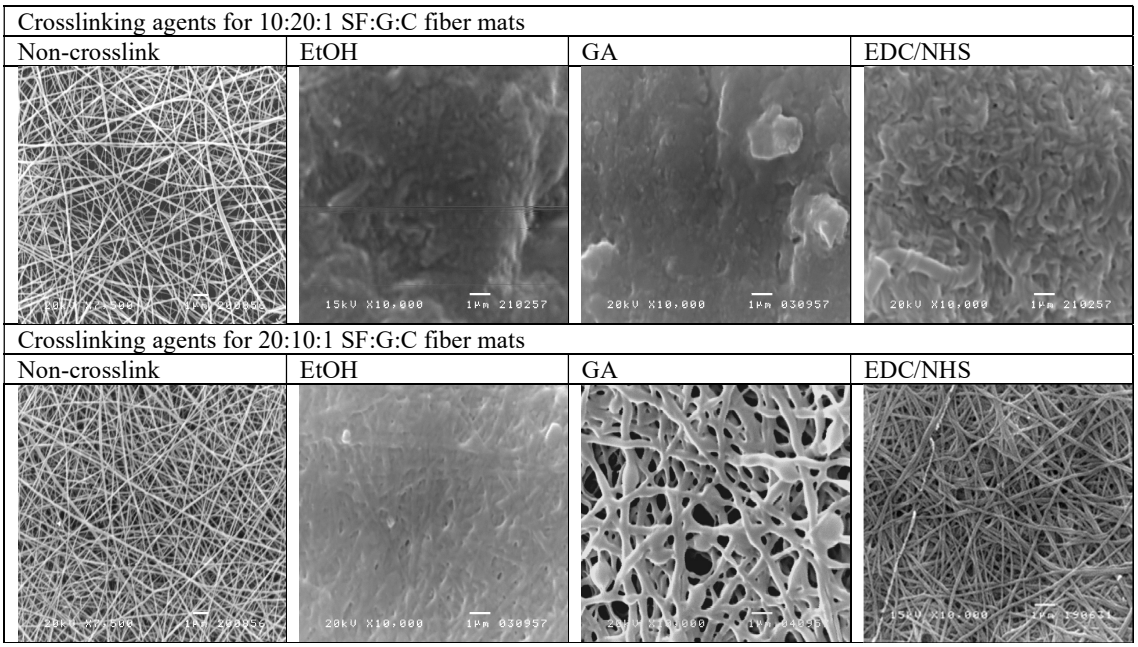


Figure 3. SEM micrograph of the SF:G:C electrospun fiber before and after crosslinked.

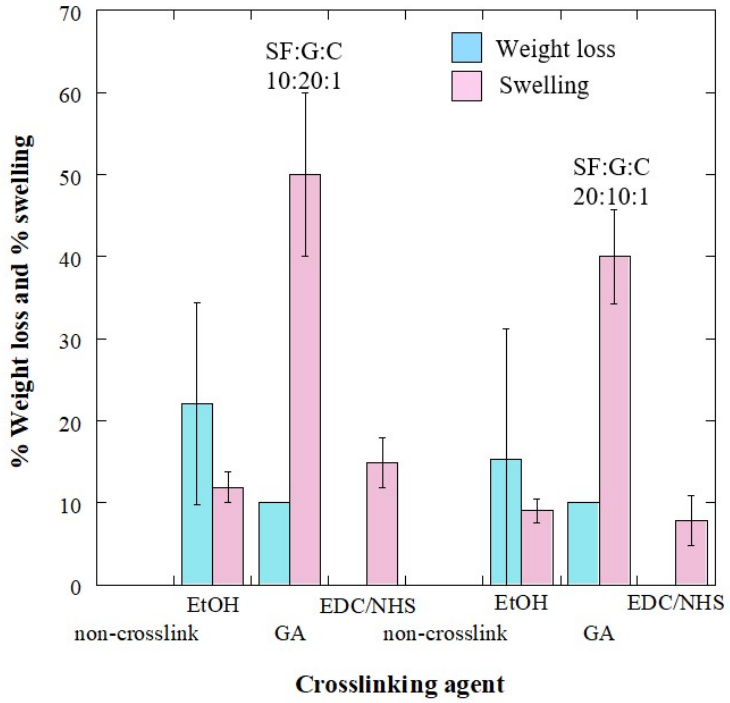


Figure 4. % weight loss and % swelling of the SF:G:C electrospun fiber mats before and after crosslinked.

No weight loss of the SF:G:C fiber mats after EDC/NHS crosslinked was measured. The EDC/NHS vapor onto nanofiber mats allowed the partially crosslinking amino and carboxyl groups and then the dipping in EDC/NHS was to crosslink the residue amino and carboxyl groups. EDC/NHS solution was known as an ideal crosslinking for material containing amino and carboxyl groups due to its well control of

crosslinking and low cytotoxicity [33]. Moreover, the original-liked morphology of the EDC/NHS crosslinked fiber mats were observed.

It was reported that EDC/NHS crosslinking agent provided optimum crosslinking degree for gelatin nanofiber membrane because carbodiimide could form intramolecular crosslink within a gelatin molecule or intermolecular crosslink between two adjacent gelatin molecules. The reaction between the ε-amino group and carboxylic acid groups by carbodiimide could weaken the protonation, so that the water absorption ability of gelatin membrane declined after crosslinking [34]. Then, the higher in the carboxyl groups led to the higher crosslinking degree, especially for the crosslinking with EDC/NHS [35].

The much swollen of fiber mats after crosslinked was observed for all crosslink agents of the fiber mats with greater gelatin content i.e. SF:G:C at 10:20:1. It was elearly noticed that SF:G:C nanofiber mats containing high gelatin content showed fused fibers and less porous structure after crosslinking. From the results, EDC/NHS agent which provided less fiber weight loss and swelling value of the crosslinked fiber mats was consequently selected for crosslinking the SF:G:C electrospun fiber mats for further biological characterization.

3.4 In vitro studies of cellular response to the SF:G:C nanofiber membranes

Normal human gingival fibroblasts were prepared from healthy gingival tissues from three donors. The explants were observed daily under the inverted microscope. The subculture was repeated once the populations of cells reach the high density. Cells from the fifth passage were used in the study of fibroblasts response to the SF:G:C membrane.

The HGF from different sources seeded on the SF:G:C at 20:10:1 fiber mats after EDC/NHS crosslinked was observed by inverted microscope after 24 h as shown in Figure 5. SEM micrographs in Figure 6 revealed the migration of cells into the nanofiber mats. The results indicated the biocompatible and non-cytotoxic of the SF:G:C fiber mats. The HGF growth on the SF:G:C at 20:10:1 membrane was also compared *Collprotect*® collagen commercial membrane. The cells spread on the SF:G:C fiber mat surface was comparable to that of the commercial membrane surface. Consequently, the EDC/NHS crosslinked nanofiber mats could support the cells spreading on the surface indicating that EDC/NHS crosslinked nanofiber mats have a potential as a membrane.

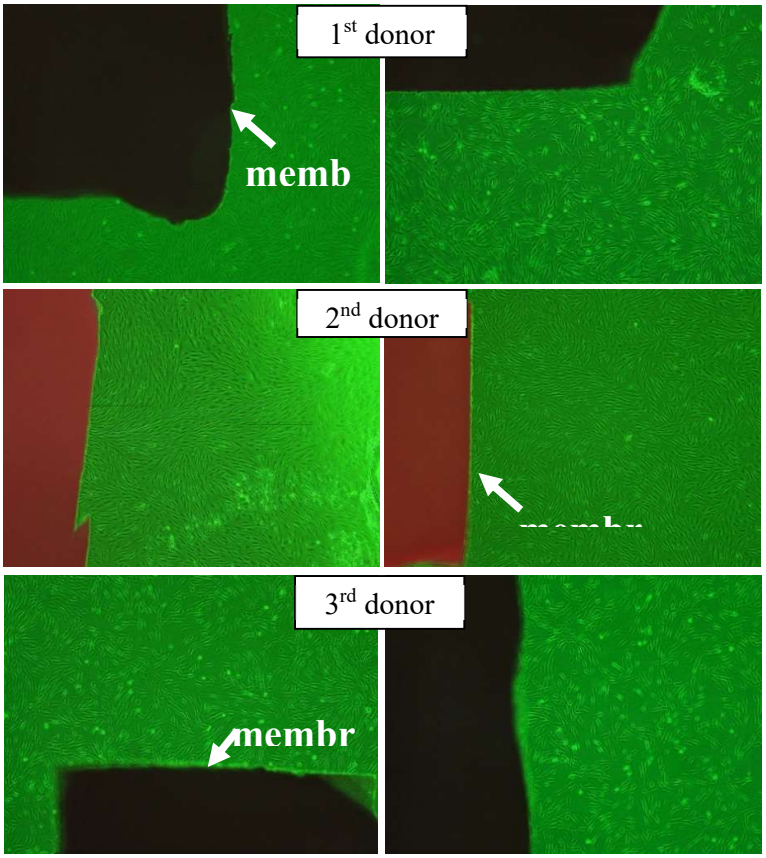


Figure 5. Normal HGF of the three donors by the inverted microscope (left: x500, right: x5,000).

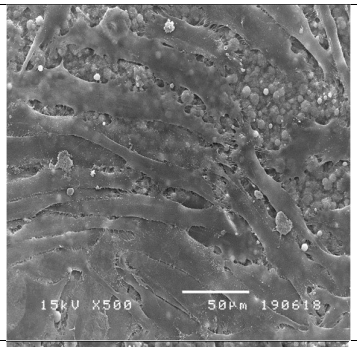
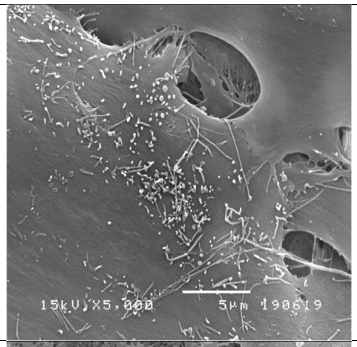
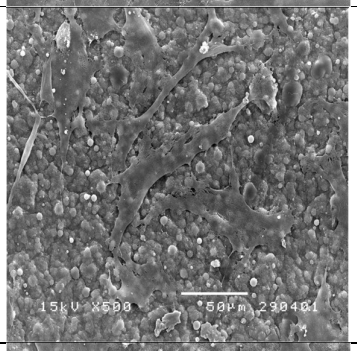
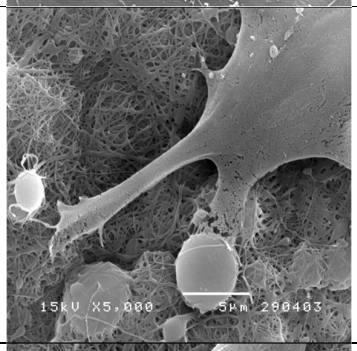
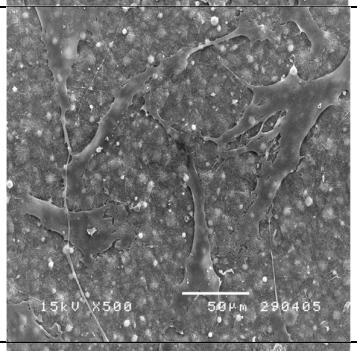
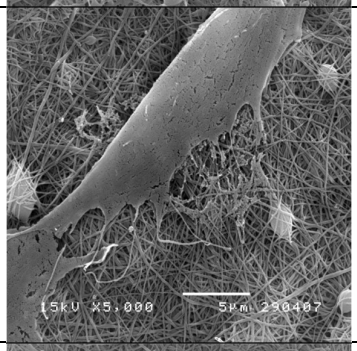
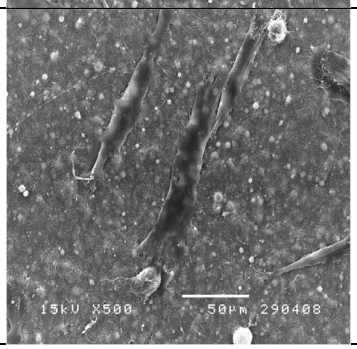
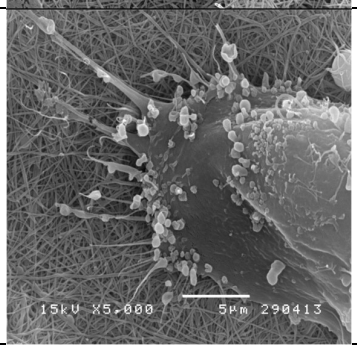
HGF from donors	SEM images	
Collprotect ® collagen commercial membrane		
1st donor		
2nd donor		
3rd donor		

Figure 6. SEM images of HGF of three donors on the SF:G:C at 20:10:1 membrane after EDC/NHS crosslinked comparing with the commercial membrane.

4. Conclusions

According to this study, the SF:G:C blended fiber mats with various ratios i.e. 10:20:0, 10:20:0.5, 10:20:1, 10:20:1.5, 10:20:2, and 20:10:1 were fabricated by electrospinning technique. The average fiber diameter of the as-spun fiber mats decreased with increasing chitosan content. A continuous and smooth nanosized-fiber without bead was obtained from the SF:G:C fiber mats at ratios of 10:20:1 and 20:10:1 by using an applied voltage of 20 kV and a distance from fiber's collector to the tip of needle of 15 cm.

The effect of three different crosslinking agents including EtOH, glutaraldehyde and EDC/NHS with

the step of crosslinking of vaporization and soaking in the solution on morphology of the fiber mats was investigated. A EDC/NHS crosslinking agent was explored to be an effective crosslinking agent because it provided the less swelling ratio and no weight loss of the fiber mats after crosslinked. A less swollen fiber was noticed in the fiber mats having higher SF content. Tensile strength of SF:G:C nanofiber having greater SF content was lower than that of with lower SF content. The results from in vitro revealed the good cell adhesion and proliferation of gingival tissues. Such results indicated the potential use of SF:G:C nanofiber mats as membrane application.

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