SIAM-WEED BASED GELATIN ELECTROSPUN SCAFFOLDS

Nor Shamsiah binti Mohamed[1], Wen Jun Ham[1], Sharaniza Ab-Rahim[2], Cheng Yee Low[1] and Ching Theng Koh[1*]

[1]Faculty of Mechanical and Manufacturing Engineering, Universiti Tun Hussein Onn Malaysia, 86400 Parit Raja, Batu Pahat, Johor, Malaysia.
[2]Dept. of Biochemistry and Molecular Medicine, Faculty of Medicine, Universiti Teknologi MARA Sungai Buloh Campus, Jalan Hospital, 47000 Sungai Buloh, Selangor, Malaysia

[*]ctkoh@uthm.edu.my

Abstract: Siam weed (Chromolaena odorata) is a traditional herb used to soothe burns and scars and potentially improve dressing and tissue-engineered construct healing ability. The use of Siam weed is mainly in the form of extract. The state of liquid extract limits the use of Siam weed compared to the state of gels and membrane. However, the development of the hybrid of Siam weed in gel and membrane form is lacking. In this study, Siam weed was harvested and made into aqua extracts. The biocompatibility of the aqua extract with various concentrations was tested using Cell Proliferation Assay. The aqua extract was then incorporated into the gelatin solution and spun into nanofibers using an electrospinning technique. The cell proliferation study shows maximum cell proliferation at the 25 μg/ml concentration. The scanning electron microscope images of the electrospun scaffolds show fibrous networks' microstructures without beads. The concentration of the hybrid solution was found to affect the morphology of the nanofibers by having diameters in the range of 160 ± 90 to 250 ± 150 nm. The Siam-weed-based gelatin electrospun scaffolds provide a native-like microenvironment and potentially improve wound healing ability for biomedical application.

Keywords: siam weed, electrospinning, gelatin, nanofiber, tissue engineering

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Introduction

Tissue-engineered skin grafts have been considered an auxiliary for replacing damaged skin due to burns and injury. There are a few current treatments such as the use of medical needling technique for improvement of scar quality, non-cultured autologous skin cell suspension for pigmentation after burn [1], small fiber neuropathy induced by resiniferatoxin to promote sensory neuropathy after third-degree burn [2] and the use of a novel chlorhexidine acetate nanoemulsion (CNE) against skin burn wound methicillin-resistant Staphylococcus aureus (MRSA) infections [3]. These treatments have been proven to improve the pigmentation of burn scars promote both mechanical and thermal hypoalgesia of the skin. CNE is a potential antimicrobial candidate for skin burn wound MRSA infections [1-3]. However, these treatments have their limitations, such as increasing the risk of failure of cell proliferation, infection, pain and wound healing problems with new scaring besides its expensive procedure [1] and prolonged healing time after treatment for example 14 days healing process after the treatment of small fiber neuropathy induced by resiniferatoxin [2]. Therefore, it is crucial to overcome these limitations by providing a practice that can meet the criteria of a successful tissue-engineered practice such as having a faster wound healing process, cheaper and can promote mechanical properties by developing three dimensional (3D) grafts to reduce the risk of failure in cell proliferation.

A standard practice of tissue engineering is seeding cells on a scaffold. The microenvironment provided by the scaffolds will conduct the regeneration and proliferation of the cells. The regeneration factors include the selection of materials, the scaffolds' microstructure, and the bioreactor that involves mechanical loading [4]. In previous studies, the natural materials used in 3D cell culture include collagen and starch [6], gelatin-chondroitin sulfate scaffold [7], cross-linked cellulose-hyaluronic acid [8] and collagen-glycosaminoglycan [9] to promote cell proliferation and regeneration. However, these natural materials were insufficient to encourage the healing process as many of these materials do not present therapeutic activities such as antibacterial and antiseptic characteristics [10]. At the same time, Siam weed extract has therapeutic activities that include antibacterial, anti-inflammatory, antioxidant and wound healing activity [11-16].

Many studies in tissue regeneration relied on two-dimensional (2D) cell culture models that fail to replicate the in-vivo cellular [17]. Conventional cell culture provides 2D space that limits cell growth, regeneration and proliferation [18]. In contrast, human tissues grow in a 3D environment surrounded by extracellular matrix and cells. The requirement of a 3D environment motivates synthetic or natural hydrogels that can create a 3D environment in cell culture platforms. Nanofibers produced by an electrospinning technique can achieve the 3D environment. Further, the nanometer fibers can also enhance their mechanical properties for successful cell proliferation and regeneration [19].

Challenges in tissue engineering graft are the difficulty in preparing the scaffolds to have therapeutic properties to promote the healing process and adequate mechanical properties [5] by developing 3D grafts imitating natural tissue. Gelatin hybrid with natural remedy was used to create a 3D environment for cell seeding [6-9]. However, these gelatin hybrids were insufficient to promote healing, especially for infected wounds due to a lack of therapeutic properties [10]. Besides, gelatin has poor mechanical properties that limit its implementation as a dressing product [10].

In this research, Siam weed has been added to gelatin to provide therapeutic properties for promoting the proliferation process. The Siam weed-based hydrogel was spun into nanofibers to enhance its mechanical properties using an electrospinning technique. This study aims to develop a Siam weed extract-gelatin crosslinked electrospun scaffold with therapeutic properties from Siam weed and good mechanical properties such as possessing excellent fiber diameter using electrospinning technique to create 3D tissue-engineered skin grafts.

Materials and Methods

Siam weed extract. Siam weed plants were collected at 1.9816° N, 102.8784° E, Parit Sulong 85300 Johor Darul Takzim, Malaysia. The preparation method of Siam seed powder was based on a previous study [20]. After washing with running tap water, the collected plants were air-dried and stored in air-tight bottles. The plants were then dried in a hot air oven at 60 °C for 24 hours before being grounded with a fine grinding planetary ball mill machine (Deco, China) to produce powders.

Two types of extracts including ethanol and aqueous extracts were prepared. For Siam seed ethanol extract, the powder was extracted with 95% v/v ethanol with a ratio of 1:10 and stirred at 120 rpm for 12 hours [21]. The mixture was filtered using Whatman filter paper No. 1 and concentrated using the double boil technique at 65 °C for 24 hours. Dark green extracts were obtained and stored in an air-tight bottle at 4 °C before testing.

Siam weed aqueous extract was prepared by stirring 600 ml of distilled water with 100 g Siam weed powder for 12 hours. The mixture was then filtered using Whatman No.1 filter paper (Merck, Germany) and concentrated using the double boil technique for 3 hours. The extract was stored in an air-tight bottle at 4 °C until testing. The aqueous extract was diluted into 5 different concentrations: 25, 50, 100, 150 and 200 µg/ml.

Siam weed based gelatin solution preparation. The gelatin fibrous scaffolds were produced by following the method of Chung et al. (2018). A 15 wt% of gelatin was dissolved in a solvent consisting of acetic acid (Sigma Aldrich, UK) and water with a ratio of 9:1. The mixed solution was stirred at room temperature overnight [22]. Siam weed aqueous extract was mixed in gelatin solution using a solvent system of 90% aqueous acetic acid (Sigma Aldrich, UK). A 10 wt% gelatin was dissolved in a solvent system of acetic acid and Siam weed extract with a ratio of 9:1 [23]. Four concentrations of Siam weed based gelatin used for electrospinning were 20 µg/ml, 25 µg/ml, 30 µg/ml, and 35 µg/ml. The solutions were then stirred for 2-3h using a magnetic stirrer at room temperature.

Production of electrospun meshes. The solution was then loaded in an electrospinning machine to produce nanofibers. The electrospinning machine consisted of an infusion pump, a high voltage power supply, and a stationary metal plate collector. The solutions were loaded into a 5 ml syringe with a 23 g needle in the electrospinning machine. Both aqueous and ethanol Siam weed extracts were used for the production of electrospun fibers. The processing parameters were kept constant where the feed rate was 0.06 ml/hr, the voltage was 15 kV and the working distance was 12 cm [22]. After electrospinning of 4 hours, the fibrous scaffolds collected were dried in a desiccator for at least 24 hours to remove residual solvent.

Crosslinking of electrospun fibers. Crosslinking was done by exposing the mesh in the saturated vapor of glutaraldehyde (GTA) (Merck, USA) for 5 hours. 5% concentration aqueous GTA was prepared by mixing up 1ml of distilled water and 9ml of GTA. After electrospinning, 25 µg/ml Siam weed-gelatin fibrous scaffolds samples were placed into a glass chamber containing aqueous GTA solution at the temperature of 40 °C for 5 hours.

Characterization: morphology. The electrospun scaffolds were gold-coated for 60 seconds and visualized in scanning electron microscopy (SEM, Hitachi SU1510, Japan). The fiber diameters Ø in SEM images were quantified using ImageJ (NIH, USA). A minimum of 4 measurements were taken to determine the average fiber diameter for each image.

Degradation test of siam weed-gelatin crosslinked scaffolds. The degradation of Siam weed-based electrospun scaffolds was examined by measuring the weight loss over three weeks. The sample was cut into 2.5 x 2 cm^2 and initial weight (W_i) was measured. The samples were then placed in 10 ml of phosphate buffer saline (PBS) solution at 37 °C. At the fixed time intervals of 1, 2 and 3 weeks, the samples were collected and washed with distilled water to remove any residuals. Excess water was removed by using filter papers. The samples were placed in the oven to dry at 37 °C until a constant mass was achieved. The final weight (W_f) of the samples was then measured. The PBS solution was replaced weekly. The weight loss (WL) was evaluated using Equation (1). The initial weight (W_i) was taken before degradation while the dry weight (W_f) was taken after degradation.

$$WL\ (\%) = \left(\frac{W_i - W_f}{W_i}\right) \times 100 \quad (1)$$

Cell proliferation assay. Chondrocytes were cultured in Siam weed extract with a concentration of 25, 50, 100, 150, and 200 mg/ml. The procedures used in the previous study were followed. Briefly, growth media was prepared using Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS) solution, and Penicillin streptomycin. A complete culture media was prepared using 400 ml DMEM, 40 ml of FBS solution, and 4 ml Penicillin Streptomycin. The media was kept at 20 °C until use. Next, the chondrocytes cells were swirled in a water bath until thawed. The cells were then put into a T25 flask and allowed to confluence up to 80% before treatment. Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega, USA) was used to investigate the number of viable cells in proliferation on Siam weed aqueous extract. Absorbance at 490 nm was recorded in an enzyme-linked immunosorbent assay (ELISA) plate reader.

Results and Discussion

Morphology of electrospun fibers. Figure 1 shows the SEM images of Siam weed-based gelatin nanofibers produced by the electrospinning technique. The morphology of Siam weed-based fibers shows a similar random fibrous network compared to gelatin electrospun scaffolds. The electrospun Siam weed ethanol extract-based gelatin scaffold has a low electrospinnability and beads appearance in the microstructure (Figure 1(b)). In contrast, aqueous extract-based gelatin shows the microstructure of fibrous networks without beads. The aqueous extract-based electrospun scaffolds were further treated by the crosslinking process. Their SEM images show the changes in morphology. The pore size of cross-linked scaffolds (Figure 1(d)) had a smaller fiber diameter of 150 ± 10 nm. The non-crosslinked scaffolds have an average pore diameter of 630 ± 60 nm (Figure 1(c)).

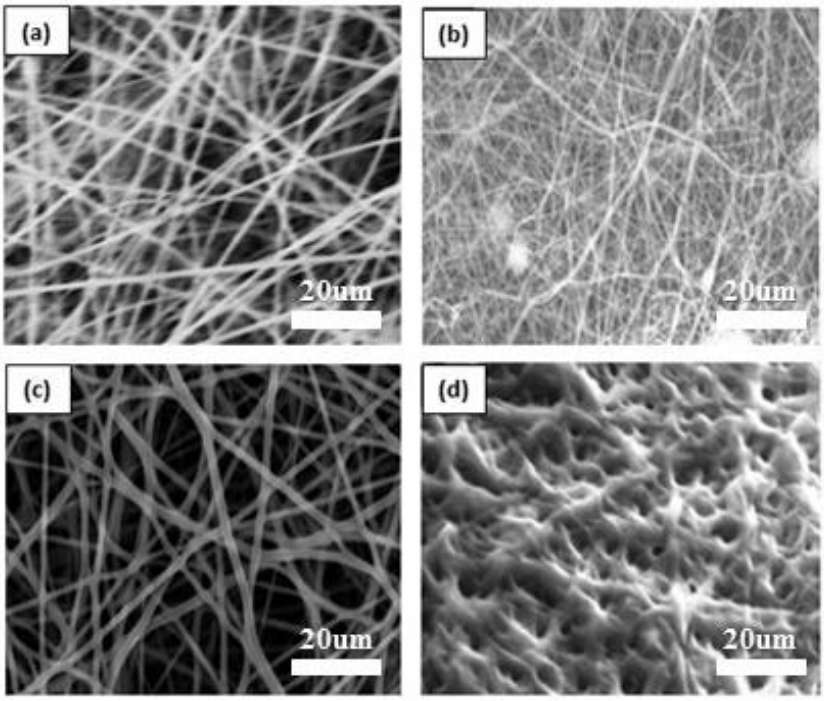

Figure 1. SEM images of (a) gelatin, (b) Siam weed ethanol extract-based gelatin, (c) Siam weed aqueous extract-based gelatin and (d) cross-linked Siam weed aqueous extract-based gelatin aqueous electrospun scaffolds.

Further, the effect of Siam weed aqueous extract's concentration on the electrospinning process was also investigated. Figure 2 shows the SEM images of the morphology of electrospun scaffolds with the concentrations of 20 µg/ml, 25 µg/ml, 30 µg/ml and 35 µg/ml. All scaffolds had the morphology of fibers without the formation of beads. The corresponding quantitative analysis of the fiber diameter of these SEM images is shown in Table 1.

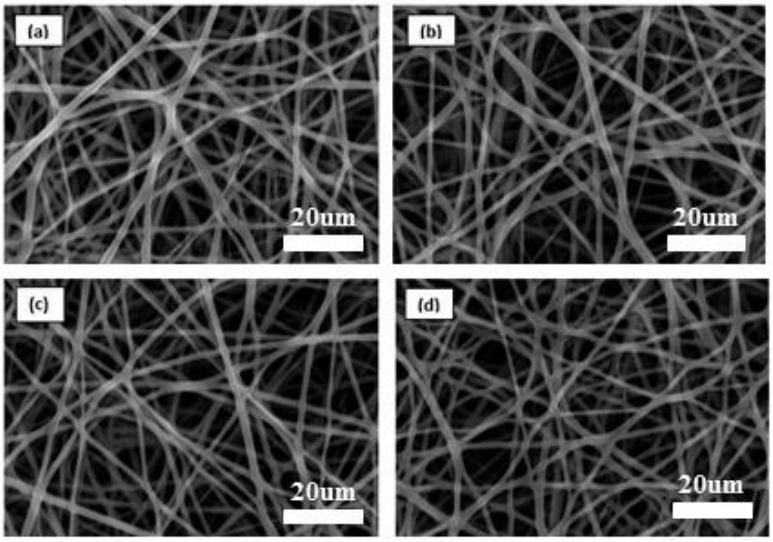

Figure 2. SEM images of Siam weed based gelatin electrospun scaffolds with concentration of (a) 20 µg/ml (b) 25 µg/ml (c) 30 µg/ml and (d) 35 µg/ml

Table 1. Effect of the concentration of Siam weed aqueous extract to the fiber diameters of the electrospun scaffolds

Concentration Siam Weed Aqueous Extract (µg/ml)	Average Fiber Diameter (nm)
20	160 ± 90
25	210 ± 90
30	230 ± 150
35	250 ± 150

The feasibility of electrospinning Siam weed ethanol extract-gelatin solutions and Siam weed aqueous extract-gelatin solutions with varying Siam weed aqueous extract concentrations were developed. The high Siam weed aqueous extract concentration provided the necessary viscosity for electrospinning. Instead of Siam weed ethanol extract, Siam weed aqueous extract-gelatin was suitable for electrospinning Siam Weed-based gelatin nanofibers. Without beads, all Siam weed aqueous extract-gelatin electrospun scaffolds with varying concentrations were successfully electrospun. The diameters of the fibers increased by the concentration of Siam weed aqueous extract. Variations of solution age are an additional factor that should be addressed when electrospinning. These solutions were electrospun to test the morphology of the fiber scaffolds, as literature shows that the electrospun fiber diameter varies with solution viscosity [24-25].

Degradation of electrospun scaffolds. The degradation of cross-linked aqueous extract-gelatin cross-linked scaffolds was examined by measuring the weight loss profile over three weeks (Figure 3). The degradation of the cross-linked scaffolds was found fast in the first week, reaching a weight loss of 8%, followed by a slower and steady loss of weight over the following weeks.

After crosslinking, the fibers of crosslinked 25 µg/ml Siam weed aqueous extract-gelatin expanded and caused a decrease in the size of inter-fibrous pores. The color change of

the cross-linked scaffolds was caused by the formation of aldimine linkages (-CH=N-) between the free amino groups of lysine or hydroxyl lysine amino acid residues of the protein and the aldehyde groups of GTA [26]. The release of Siam weed compounds from the scaffold caused a substantial loss of weight significant weight loss. The weight loss from the Siam weed aqueous extract-gelatin cross-linked scaffold appeared to stabilize after this phase. This characteristic allows the Siam weed gelatin fibrous scaffolds to be used as a wound dressing for dried and exuding wounds.

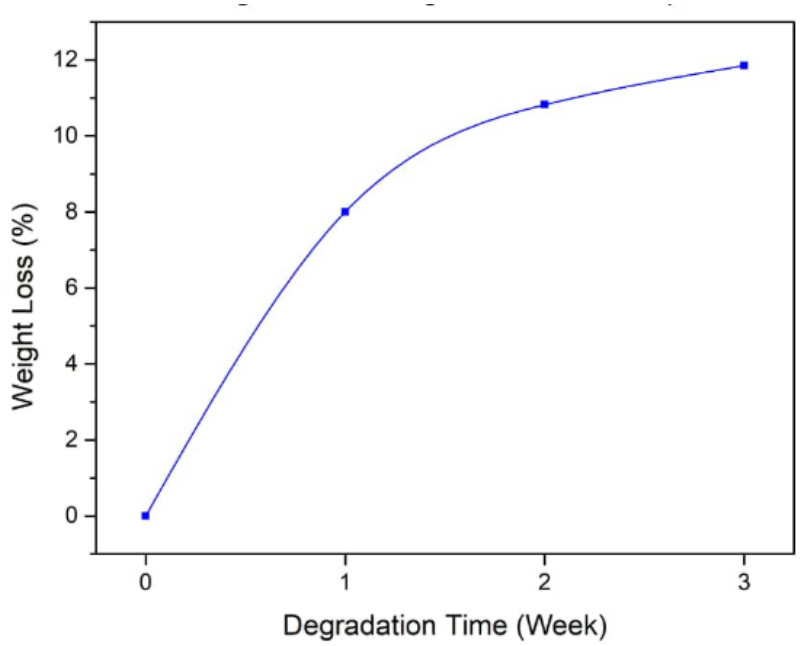

Figure 3. Weight loss profiles measured over three weeks.

Cell proliferation. Figure 4 shows that Siam weed aqueous extract significantly stimulated cell proliferation with a strong response at 25 ug/ml concentration followed by 50 ug/ml. The cell viability in the extract with a concentration of 100 ug/ml was insignificant compared to the control. The cells started to die off at 150 ug/ml and 200 ug/ml doses.

The analysis of cell proliferation is essential to determine the cellular responses to the extract. Siam weed aqueous extract has been shown to promote cell proliferation significantly good viability at 25 µg/ml concentration, resulting in optimum cell proliferation. The findings show that the Siam weed extract with a concentration of 25 µg/ml is biocompatible.

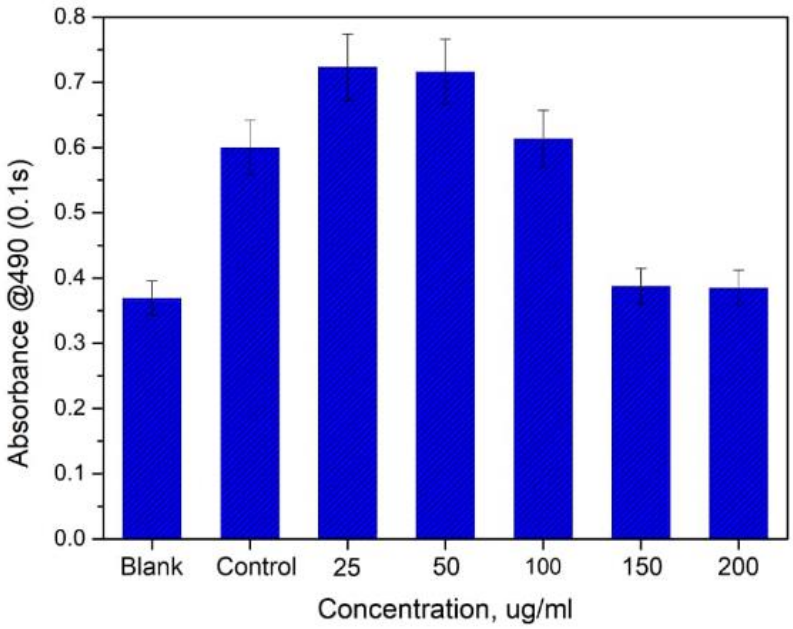

Figure 4. Effect of Siam weed aqueous extract concentration on the absorbance vs. concentration.

Conclusion

Siam weed aqueous extract with a concentration of 25 μg/ml was biocompatible and used for further study. The extract was successfully incorporated in gelatin solution to produce nanofiber using an electrospinning technique. Unlike aqueous extract, ethanol extract had the poor spin ability and showed bead formation. The degradation of the Siam weed-based electrospun scaffolds was stable approximate 12% of weight loss over 3 weeks. Siam weed-based gelatin nanofibers show potential for skin tissue engineering and wound dressing applications.

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Author Contributions

All authors contributed toward data analysis, drafting and revising the paper and agree to be accountable for all aspects of the work.

Disclosure of Conflict of Interest

The authors have no disclosures to declare

Compliance with Ethical Standards

The work is compliant with ethical standards

Reference

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