Morphology of Keratinocyte (HaCaT) Cells on Electrospun Nanofibers of Polyhydroxyalkanoate Biopolyester Containing *Zingiber cassumunar* (Plai Oil)

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Abstract

Nanofibers have been used in wound dressings and protective clothing for biomedical applications. Electrospinning is a fiber production method in which electric force is applied to draw charged threads of polymer solutions to non-woven nanofibers. Plai is a traditional Thai medicine for musculoskeletal pain relief and other ailments. Thus, incorporation of Plai into electrospun Polyhydroxyalkanoate (PHA) nanofibers to control release of bioactive components can enhance the value of Asian herbal therapeutics. This research focuses on the production of electrospun nanofibers of PHA biopolymer loaded with Plai Oil and investigates the characteristics of fibers and their impacts on Keratinocyte Cells (HaCaT) morphology. PHA nanofiber loaded with 40% Plai Oil was fabricated by the electrospinning method. The diameters of the samples were investigated by the SEM micrograph. The unloaded Plai Oil and 40% loaded Plai Oil specimens were cultured with HaCat Cells. After 48 hours of incubation, the morphology of the cells were fixed and observed by SEM. The smooth and nonwoven fibers were obtained from the electrospining method. The result indicated that Plai oil component was blended in the fiber structure that expanded the diameter sizes of the fibers. After the incubation of both unloaded and 40% loaded Plai Oil in PHA nanofibers with HaCat Cells, the result showed that cells could grow and attach to each other on the fibers as well as secret their extracellular matrix. By the electrospinning method, it is possible to fabricate PHA nanofibers in cooperated with Plai Oil and all specimens exhibiting biocompatibility property which can be used in biomaterials applications.

Keywords**:** *electrospinning, nanofiber, plai oil, polyhydroxyalkanoate*

บทคัดย่อ

ปัจจุบันได้มีการนำวิธีอิเล็กโตรสปินนิงมาใช้ในการผลิตเส้นใยนาโนสำหรับใช้งานวิจัยทางการแพทย์โดยเฉพาะในวัสดุชีวภาพ น้ำมันไพลสกัดจากพืชสมุนไพรถูกนำมาใช้ในการแพทย์แผนไทยเพื่อลดอาการบรรเทาปวดของกล้ามเนื้อ การผสมไพลเข้าไปในเส้นใยนาโนของพอลิไฮดรอกซีอัลคาโนเอตสามารถสร้างแผ่นฟิล์มชีวภาพที่สามารถควบคุมการปลดปล่อยสารว่องไวทางชีวภาพได้ จึงนับว่าเป็นสิ่งที่เพิ่มมูลค่าผลิตภัณฑ์ได้ ดังนั้นงานวิจัยนี้จึงมีวัตถุประสงค์เพื่อศึกษาคุณลักษณะของเส้นใยนาโน PHA ที่ผสมไพล ทดสอบความเป็นพิษและการเข้ากันได้ทางชีวภาพ โดยการพิจารณาจากสัณฐานวิทยาของเซลล์ที่เพาะเลี้ยงบนเส้นใยที่เผลิตขึ้น ในการทดลองนี้ได้เตรียมเส้นใยสองชนิดคือเส้นใยนาโนพอลิไฮดรอกซีอัลคาโนเอตแบบไม่ใส่น้ำมันไพล และใส่ไพลในสัดส่วน 40 เปอร์เซ็นต์ โดยการขึ้นรูปด้วยวิธีอิเล็กโตรสปินนิง จากนั้นทดสอบความเป็นพิษและความเข้ากันได้ทางชีวภาพโดยนำเซลล์ฮาคัตซึ่งเป็นเซลล์ไลน์ชนิดเคราติโนไซต์ไปเพาะเลี้ยงบนเส้นใยที่ผลิตขึ้นเป็นเวลา 48 ชั่วโมง จากนั้นทำการตรึงเซลล์แล้วศึกษาสัณฐานวิทยาของเซลล์บนเส้นใยนาโนด้วยกล้องจุลทรรศน์ชนิดส่องกราด จากผลการทดลองพบว่าการใส่ไพลลงในเส้นใยส่งผลให้เส้นใยมีขนาดใหญ่ขึ้นและจากการศึกษาผลต่อเซลล์พบว่าเส้นใยทั้งสองตัวอย่างไม่เป็นพิษกับเซลล์ โดยการพิจารณาจากการเติบโต การยึดเกาะของเซลล์บนเส้นใย และยังพบว่าเซลล์มีการหลั่งเมตริกซ์นอกเซลล์ออกมาจึงเป็นการยืนยันว่าวัสดุมีความเข้ากัน ได้ทางชีวภาพ ซึ่งมีศักยภาพในการนำไปประยุกต์ใช้งานทางการแพทย์

คำสำคัญ: *อิเล็กโตรสปินนิง เส้นใยนาโน น้ำมันไพล พอลิไฮดรอกซีอัลคาโนเอต*

1. Introduction

Nanofibers, a fabrication, have been investigated for their beneficial properties as wound dressings, reinforcement fibers in composites, protective clothing, filtration, and biomedical devices due to their highly specific surface area that plays a major role in controlling drug release, promoting bioactivity and biocompatibility, and highly increasing nanoporous strength/elasticity (Ko and Wan, 2014).

Electrostatic spinning or electrospinning technique is a process in which electrical force is used as a mean to create charged liquid jet from liquid surface and stretch into thin charged liquid jet that affects a dry fiber at countered or grounded collector (Dalton et al., 2007; Reneker et al., 2008; Thompson et al., 2007; Yarin et al., 2001). Mostly material is being investigated and numbers of successful spinning case are drastically increased. For processing of non-woven nanofibrous, electrospinning is an electrostatic technique according to simply devices and universal using, a variety of characteristic modification capability that play a role in advance medical research. Nanofiber scaffold with three-dimensional architecture extracellular matrix mimic and biocompatibility of polymer are key success to improve the function or treatment damaged tissue or organ (Gaharwar et al., 2014; Sun et al., 2014).

In this work, we focused on the nanofiber production from Polyhydroxyalkanoate (PHA) containing Thai traditional herbal extract of Plai oil. Plai, known as *Zingiber cassumunar Roxb* (the family *Zingiberaceae*), is a perennial herb which has been used for treatment of inflammation and muscle and joint problems. Plai essential oil (distilled oil) contains a lot of volatile compounds: sabinene, terpinene, terpinen-4-ol, and DMPBD (Giwanon et al., 2000; Pithayanukul et al., 2007). PHA is a natural bio-based polyester formed and accumulated by many microbial species as intracellular carbon (Anderson and Dawes, 1990; Lenz and Marchessault, 2004; Luzier, 1992). The PHA polymers can contain or be modified to include other molecules, such as bioactive and detectable compounds, surface active agents, other degradable or non-degradable polymers, and materials used to modify the mechanical properties of the PHAs, such as plasticizers, fillers, and binders. This may be useful for controlling tissue regeneration or other processes that are affected by the concentration of specific agents. The first generation of Plai, used for musculoskeletal pain relief, was in the form of crude plai oil extracted by deep-frying method. When adapted, then, it was in the form of herbal balls more convenient in use with a longer shelf life than crude oil. Plai cream is the third generation, consisted of Plai essential oil or Plai deep fried oil. Therefore, a combination of electrospun nanofiber and Plai essential oil can become a novel generation of Thai herbal products (Suksaeree et al., 2015). This study figures out a suitable proportion of PHA to Plai oil which perform the characterization. This research aimed to investigate the characterization and cell morphology on the nanofibers of PHA copolymer containing 3-hydroxybutyrate (3HB) and 4-hydroxybutyrate (4HB) monomer units which were loaded by a high amount of Plai oil extract at 40% to confirm the growth and toxicity of skin keratinocyte cells.

2. Objectives

The objective of this research focused on the production of electrospun nanofibers of PHA biopolymer loaded with Plai Oil and an investigation of the characteristics of fibers and their impacts on Keratinocyte Cells (HaCaT) morphology.

3. Materials and methods

3.1 Materials

3.1.1 Polyhydroxyalkanoate (PHA) copolymer, purchased from Ecomann Biotechnology (Shandong, China), containing about 88% of 3-hydroxybutyrate and 12% of 4-hydroxybutyrate

3.1.2 Plai distilled oil from Thai-China Flavours and Fragrances Industry Co., Ltd (Thailand)

3.1.3 Dichloromethane (DCM, analytical grade) from Sigma Aldrich (USA)

3.2 Preparation of PHA-Plai oil electrospun nanofibers

Polymer solutions were prepared by dissolving PHA (5%, 8%, and 10% w/v) in dichloromethane. A PHA concentration of 8% w/v was chosen as optimal for Plai oil loading. Plai oil at different concentrations (5 and 40 % of Plai oil in PHA w/w) was added, in separate batches, to the 8% PHA solution prepared above. The electrospinning of PHA and PHA-plai oil were carried out using a 5 ml syringe equipped with a blunt needle (0.5 mm diameter) at 20 kV (Gamma High Voltage) and a flow rate of 1 ml/h, as shown in Figure 1. The collector was an aluminium foil plate maintained at a distance of 20 cm from the needle. The process duration time was 4 hours.

3.3 Methods of cell culture (HaCaT)

HaCaT cells, the immortal keratinocyte cell lines from adult human skin, were cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies-Gibco, Carlsbad, CA, USA) supplemented with 10% fetal calf serum (Life Technologies-Gibco), 100 mg/ml Penicilin-Streptomycin (Life Technologies-Gibco) and 250 ug/ml Amphotericin B (Sigma-Aldrich). The cells were incubated at 37°C in a humidified atmosphere of 5% CO_2. 200,000 cells were seeded onto each sample (a size of 1cm. x 1 cm.) on a 6-well plate and incubated at 37 °C for 48 hours. The qualitative evaluations of cell viability and cell attachment were done by photograph with phase contrast camera and Scanning Electron Microscope (JSM-6400, JEOL Ltd., Tokyo, Japan), respectively, with some modified procedures of previous studies (García et al., 2010; Zhao et al., 2016).

3.4 Morphology characterization

Scanning Electron Microscope (SEM) (JSM-6400, JEOL Ltd., Tokyo, Japan) was used to characterize the characteristics and sizes of electrospun fibers. Samples of the electrospun nanofibers were sputter coated with platinum. Images were captured with an accelerating voltage of 5 kV. Image J software (www.imagej.nih.gov) was used for measuring the fiber diameter. The nanosize of the nanofibers was measured by averaging the size compared with the bar on the picture.

4. Results and Discussion

Non-woven nanofibers via electrospining were processed by a high voltage on the needle and a zero voltage on the plate collector (grounded), and accumulated electrostatic on the PHA/DCM droplet was pushed by a high voltage on the needle to the collector. Therefore, the droplet became a solution jet that rapidly elongated the solution into such a “nano” size that the DCM solvent was completely evaporated before the nanosized fiber dropped on the collector as shown in Figure 1.

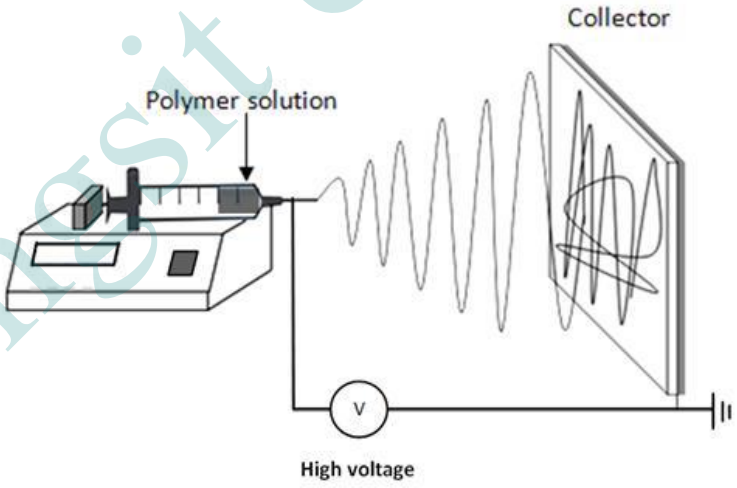
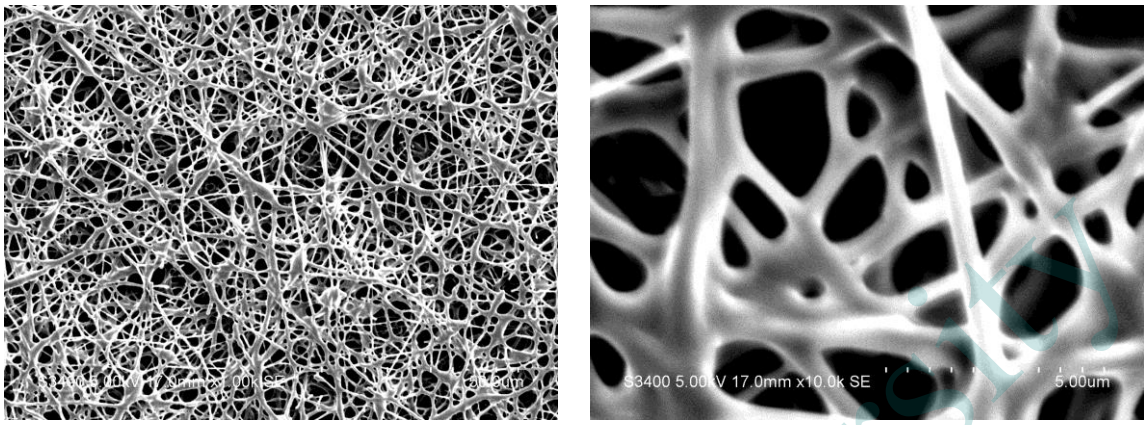


Figure 1 Electrospinning experimental set up

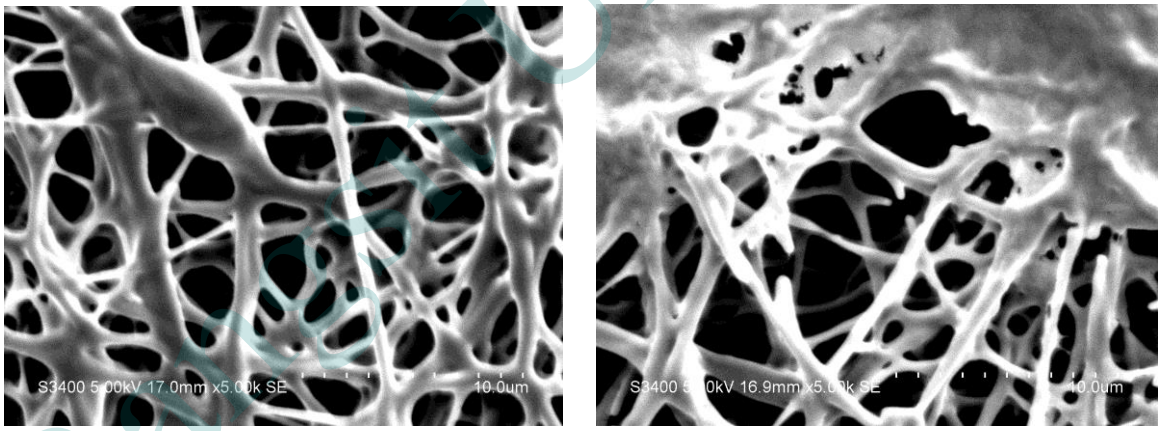
In this study, the 8%PHA solution was selected, as in our previous study, the 8%PHA nanofiber via electrospinning exhibited a smooth cylindrical, uniformity and diameter less than 1 µm of fibers formation, as shown in Figure 2 detailing the SEM images of 1000x and 10000x magnification. 40% Plai oil loaded with nanofibers exhibited smooth cylindrical and uniformity as well; on the other hand, the diameter was 1.28±0.34 µm larger than 8%PHA (0.83±0.14 µm). This result indicated that Plai oil component blended in the fiber structure could expand the diameter size.



(a) **(b)**

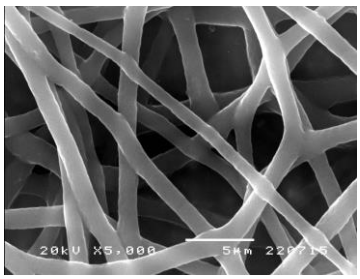
Figure 2 SEM images PHA/DCM electrospun nanofibers 8%PHA/DCM at 1,000x (a) and 10,000x (b) magnification

The biocompatibility was evaluated by culturing nanofiber specimens with HaCat, Keratinocyte cell line, for 48 hours. Figure 3 shows the morphological micrographs of 8% PHA nanofibers (a) and the seeded material with HaCat Cells (b). The result indicated that HaCat cells can successfully grow on the PHA nanofibers as shown in Figure 3b. This result showed the 8% PHA nanofiber was nontoxic.

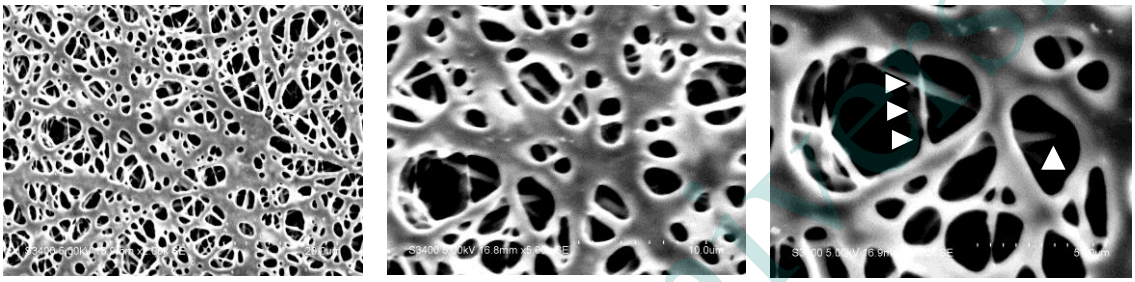


(a) **(b)**

Figure 3 SEM image of PHA/DCM electrospun nanofibers 8%PHA/DCM at 5000x (a) and SEM micrograph of HaCaT cells on the electrospun PHA nanofiber in magnification of 5000x (b)



(a) 5000X



(b) 2000X **(c)** 5000X **(d)** 8000X

Figure 4 SEM micrograph of unseeded cells (a) and those cultured with HaCaT cells on PHA loaded with 40% Plai oil in various magnifications (b, c and d)

Figure 4 shows the SEM micrographs of 40% loaded Plai Oil PHA nanofibers morphology. At 5000X of an unseeded cells sample, the orientation as same as scaffold with interconnected pores has potential to enhance the cell's growth (Figure 4a). After the culture of the samples with HaCat cells for 48 hours, the cells viability was investigated as shown in Figure 4b, 4c, and 4d. Cells could attach and spread their pseudopodia on nanofibers as well as secrete their extracellular matrix which remarks the biocompatibility of specimen. Moreover, cells could grow inside the micro pores as shown in Figure 4d (white arrows).

5. Conclusion

A 40% Plai oil loaded PHA nanofiber was fabricated using the electrospinning method. The fibers were characterized by SEM, and the diameter of 40% Plai oil loaded PHA fiber is bigger than that of 8%PHA nanofiber. After the incubation of both specimens with HaCat Cells Line, the result exhibits the biocompatibility of nanofiber materials as cells could attach on fibers and secrete their extracellular matrix. Moreover, it can be concluded that Plai Oil is a nontoxic substance when loaded into nanofibers. By electrospinning method, PHA nanofibers with a combination of Plai Oil and all specimens can be fabricated as they exhibit biocompatibility property which can be used in biomaterials applications.

6. Acknowledgements

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