THE SURFACE PROPERTIES OF THE COMPOSITE POLY(ε-CAPROLACTONE) SCAFFOLD FOR TISSUE ENGINEERING

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Thesis

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THE SURFACE PROPERTIES OF THE COMPOSITE POLY(ε-CAPROLACTONE) SCAFFOLD FOR TISSUE ENGINEERING

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ABSTRACT

Recently, scaffold for tissue regeneration has been studied intensively due to its capability to promote three dimensional cell growth. Poly(ε-caprolactone) is chosen in this research due to its lack of toxicity, good biocompatibility and good mechanical properties for soft and hard tissue and low cost. Scaffolds were prepared using electrospinning technique and solvent casting/salt leaching method. These scaffolds were then modified using collagen, chitosan, chondroitin sulfate, gelatin and glucosamine HCL to improve hydrophilicity of the surface. Scaffold morphology, water contact angle and surface roughness were examined using a Scanning Electron Microscope, contact angle goniometer, and Atomic Force Microscopy, respectively. The scaffolds were also tested for the %water uptake and enzymatic degradation rate. Fourier Transform-Infrared spectra were used to determine the residual chloroform in the scaffolds. The scaffolds were studied for possible use in tissue engineering by in vitro hepatocyte cell culturing. Electrospinning is an alternative technique to fabricate scaffolds. However, solvent casting/salt leaching is the attractive method due to its easy processing and controllable porosity. The results showed that blending organic additives into polymer solutions can change surface morphology. The surface morphology affects surface hydrophobicity. Collagen showed a significant improvement of surface wettability. The other properties such as swelling ratio and degradation rate showed no significant differences between each sample. The obtained scaffolds can be used as a template for three dimensional tissue growth. It seems that cells can attach and proliferate well on all the composite scaffolds.

KEY WORDS: POLY(ε-CAPROLACTONE)/ELECTROSPINNING/SOLVENT CASTING -SALT LEACHING/ORGANIC ADDITIVES/SURFACE HYDROPHOBICITY

81 P.

การศึกษาคุณสมบัติทางพื้นผิวของเยื่อโครงสร้างผสมโพลีคาโปรแลคโตนสำหรับวิศวกรรมเนื้อเยื่อ
(THE SURFACE PROPERTIES OF THE COMPOSITE POLY(ε-CAPROLACTONE) SCAFFOLD FOR TISSUE ENGINEERING)

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บทคัดย่อ

ปัจจุบันเยื่อโครงสร้างสำหรับสร้างเนื้อเยื่อทดแทนกำลังได้รับความสนใจอย่างกว้างขวาง ทั้งนี้เนื่องจากความสามารถในการสร้างเนื้อเยื่อในลักษณะสามมิติ ในการศึกษานี้เลือกใช้โพลีคาโปรแลคโตนเนื่องจากมีความความเป็นพิษน้อย มีความเข้ากันทางชีวภาพที่ดี มีคุณสมบัติเชิงกลที่เหมาะสมสำหรับทั้งเนื้อเยื่ออ่อน เนื้อเยื่อแข็งและมีราคาถูก เยื่อโครงสร้างถูกเตรียมโดยวิธี electrospinning และ solvent casting/salt leaching และมีการศึกษาการปรับปรุงคุณสมบัติทางพื้นผิวของเยื่อโครงสร้างโดยการเติมสารอินทรีย์ ได้แก่ collagen, chitosan, chondroitin sulfate, gelatin and glucosamine HCL เพื่อให้ได้พื้นผิวที่มีคุณสมบัติชอบน้ำ การศึกษาคุณสมบัติของเยื่อโครงสร้างประกอบด้วยการวิเคราะห์ภาพถ่ายกล้องจุลทรรศน์อิเลคตรอนชนิดส่องกราด, การวัด water contact angle โดยใช้ contact angle goniometer, การวัดความขรุขระโดยอาศัย Atomic Force Microscopy, ความสามาถในการอุ้มน้ำ, อัตราการย่อยสลาย, การหาสารตกค้างของตัวทำละลายในเยื่อโครงสร้างโดยใช้ Fourier Transform Infrared spectrum และการศึกษาความเป็นไปได้ที่จะใช้เยื่อโครงสร้างในวิศวกรรมเนื้อเยื่อโดยดูการเจริญเติบโตของเซลล์ตับในหลอดแก้ว

จากการศึกษา พบว่า electrospinning เป็นวิธีที่ใช้สร้างเยื่อโครงสร้างได้แต่ solvent casting/salt leaching เป็นวิธีที่ง่ายกว่าและสามารถควบคุมความพรุนได้ และการเติมสารอินทรีย์ลงในสารละลายโพลิเมอร์สามารถเปลี่ยนลักษณะของพื้นผิวได้ ซึ่งการเปลี่ยนแปลงลักษณะของพื้นผิวมีผลต่อ wettability โดยที่ collagen เป็นสารอินทรีย์ที่ให้ผลการเปลี่ยนแปลงที่เห็นได้ชัดที่สุดในการปรับปรุงความชอบน้ำ ส่วนคุณสมบัติอื่นๆ เช่น ความสามารถในการอุ้มน้ำและอัตราการย่อยสลาย ผลการทดลองแสดงให้เห็นว่า ไม่มีความแตกต่างที่เห็นได้ชัดในแต่ละตัวอย่าง ทั้งนี้ เยื่อโครงสร้างที่ได้สามารถนำไปใช้ในการสร้างเนื่อเยื่อแบบสามมิติได้ ผลการทดลองแสดงให้เห็นว่า เซลล์ตับสามารถเกาะและเจริญเติบโตบนเยื่อโครงสร้างผสมทั้งหมดได้

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LIST OF SYMBOLS AND ABBREVIATIONS

%	Percent
/	Per
3-D	Three dimensions
AFM	Atomic force microscopy
ATR-FTIR	Attenuated total reflection-fourier transform infrared spectroscopy
AY	Candida cylindracea lipase bioreactor system
cm	Centimeter
CO_2	Carbondioxide
d	Distance between the needle tip to the ground collector
DCM	Dichloromethane
DMF	*N,N*-dimethylformamide
ECM	Extracellular matrix
EM	Electromagnetic
etc.	Et cetera (Latin), and so fort
FTIR	Fourier transform infrared spectroscopy
GAG	Glycosaminoglycan
h	Hour
HCL	Hidrochloride
i.e.	id est (Latin), that is
IR	Infrared
kV	Kilovolt
M	Mole
MC	Methylene chloride
MEFs	Mouse embryonic fibroblasts
ml	Milliliter
MW	Molecular weight
N/A	Not applicable

LIST OF SYMBOLS AND ABBREVIATIONS (continued)

NaCl	Sodium chloride
NH_2	Amine group
nm	Nanometer
P(LLA-CL)	Poly(L-lactic-co-ε-caprolactone)
PAA	Polyacrylic acid
PBS	Phosphate buffer solution
PBT	Polybutylenes teraphthalate
PCL	Poly(ε-caprolactone)
PEGT	Poly(ethylene glycol)-teraphthalate
PEO	Polyethylene oxide
PGA	Polyglycolic acid
PHA	Polyhydroxyalkanoate
PLGA	Poly(lactic-co-glycolic acid)
PLLA	Polylactic acid
PP	Porcine pancreatic lipase
PS	Pseudomonas lipase
PTFE	Polytetrafluoroethylene
PU	Polyurethane
PVA	Polyvinyl alcohol
r	Surface roughness
Ra	Roughness
RGD	Arginine-glycine-aspartate
ROBS	Rotational oxygen-permeable
SD	Standard deviation
SEM	Scanning electron microscope
TCA	Tricarboxylic acid
USFDA	United States Food and Drug Administration
UTM	Universal testing method
w/v	Weight by volume

LIST OF SYMBOLS AND ABBREVIATIONS (continued)

Wa	Weight of the scaffold after enzymatic test
WAXD	Wide angle X-ray diffraction
Wb	Weight of the initial scaffold before enzymatic test
Wd	Weight of the dry scaffold
Ws	Weight of the swollen scaffold
γ_{lv}	The interfacial tension between a solid and the liquid drop
γ_{sl}	The force balance between liquid-vapor surface tension of a liquid drop
γ_{sv}	The surface energy
θ	Contact angle
θ_w	Wenzel's angle
μm	Micrometer

CHAPTER I
INTRODUCTION

1.1 Background

Tissue engineering is a new and exciting field of study, this study involes with procedures to reconstitute living cells and other natural substances in form of tissues or organs which can be used to repair, maintain or enhance body structure and function. It is usually composed of two important components: a group of appropriated cells and a scaffold which the cell can grow on. Scaffold is three-dimensional structures that allow cells to be transfer and attach during healing process. It serves as a template for cell adhesion, proliferation, and differentiation into a specific tissue. Two type of scaffold are commonly used; non-absorbable and absorbable which the later is more preferred.

Generally, a scaffold must have a highly porous structure with interconnected pores and a large surface area that can enhance cell adhesion. These conditions will provide a better environment for the cells to attach. The scaffold must be processed into a form (i.e. film, bead, or porous sheet) that can provide the appropriated environment for the engineered cells to develop into a fully functional tissue. The preparation techniques can be adopted from typical polymer casting technique such as solution casting, phase separation, electro-spinning, or solvent-casting associated with particle leaching, etc.

Many of biodegradable polymers such as polycaprolactone (PCL), poly(lactic-co-glycolic acid) (PLGA), polyethylene glycol, polyvinyl alcohol, and polyurethane are investigated. However, PCL is one of the attractive biodegradable polyesters in this study due to its availability. It is semi-crystalline linear resorbable aliphatic

polyester. It has the susceptibility of aliphatic ester linkage to hydrolysis. The products generated are either metabolized via the tricarboxylic acid (TCA) cycle or eliminated by direct renal secretion resulting in the lacking of toxicity [25]. This biodegradable polymer has good mechanical properties (PCL membranes formed after dissolution in chloroform show elongation up to 1000% before break) [37] which allow us to use as the soft or hard tissue scaffolds. It also has good physical properties such as; low melting point (~58-60 ˚C) which can be process easily, slow degradation rate due to its high crystallinity, and hydrophobicity, which can be used in various applications. Moreover, it has low cost when compared to other biodegradable polyesters. Thus, this material is suitable for biomedical applications as approved by United States Food and Drug Administration (US FDA) for medical and drug delivery devices.

A large number of PCL applications have been investigated especially in the drug delivery system and musculoskeletal tissue engineering such as cartilage and bone. Although PCL has shown very promising in biomedical applications, the properties of PCL solely may not suitable for some applications. Therefore, some studies have been carried out to compensate the requirement of each application. For example, PCL is blended with other biodegradable polymer such as polylactic acid (PLLA) to represent the vascular structure for blood vessel in tissue engineering [45]. Some research prepared composite PCL with the organic substances to improve its biomechanical properties i.e., PCL/chitosan to observe the in vitro cell culture of mouse embryonic fibroblasts (MEFs) [37], PCL/silk fibrobrin for in vitro human fibroblast culture [9], and PCL/collagen for skin tissue engineering [11].

However, PCL has been reported to have limitations of bioregulatory activity due to the lack of bioactive functional groups [9], neutral charge distribution, susceptibility to bacteria-mediated degradation [37]. These limitations can be enhanced by blending PCL with a variety of organic substances such as silk, chitosan, and collagen. Blending of PCL and organic substances provides the wide range of benefits in physicochemical properties that the easy to process applicable to synthetic

PCL and the improving in the bioactivity of cells applicable to organic substances. Alhough, the blending composite has a lot advantages as mentioned earlier, a disadvantage may found in the composite scaffold i.e. the reduction of the mechanical strength due to the decreasing in crystallinity of PCL composite. Therefore, blending synthetic PCL with organic substances is an alternative of improving the hydrophilicity of the scaffold without significant affects to its mechanical properties.

In this study, composite PCL films are prepared using solvent-casting technique. PCL is blended with collagen, chitosan, glucosamine HCL, chondroitin sulfate, or gelatin, using chloroform as a solvent. Wettability and roughness of the prepared films are characterized using contact angle goniometry and atomic force microscopy respectively. The expected results are the enhancing in surface hydrophilicity of composite films by reducing the water contact angle of the composite films compared to pure PCL film. This study will followed by composite scaffold preparation using solvent casting/salt leaching technique. The morphology, swelling test, enzymatic degradation rate of the obtained scaffolds will be studied. In addition, cytocompatibility of hepatocyte cells will be tested to prepare the biodegradable scaffold for using in tissue engineering.

1.2 Objectives

This research is conducted in order to

(1) Study the effect of fabrication techniques for PCL scaffold preparation

(2) Modify the surface properties of PCL scaffold to for tissue engineering

1.3 Scope of studies

(1) Prepare PCL scaffolds using two techniques; Electrospinning and solvent casting/salt leaching

(2) Study the effect of synthesis parameters such as solution concentration and quantity of porogen to the scaffold properties

(3) Study the effect of organics additive to the surface properties of scaffolds

(4) Investigate the surface properties of PCL and PCL composite scaffold such as the morphology using Scanning Electron Microscopy (SEM), wettability by optical water contact angle measurement using contact angle goniometer, roughness using atomic force microscopy (AFM) by the aid of scanning probe microscope controller, swelling test, enzymatic degradation test, and residual chloroform in sample using Fourier Transform Infrared Spectroscopy (FTIR)

(5) Study the cell culturing for using in tissue engineering by seeding the hepatocyte cells and assessed their ability to support cell seeding and growth in vitro

CHAPTER II
LITERATURE REVIEW

In the study on "surface properties of the composite polycaprolactone scaffold for tissue engineering", this chapter will present the relevant information and theories with importance on the scaffold study.

2.1 Background

Recently, tissue engineering is a field which immersing the application of bioscience and engineering together. It is an understanding of the development of biological substitutes that restore, maintain or enhance body function. The aim of tissue engineering is to exceed the limitation of conventional treatments such as organ transplantation and biomaterials implantation. It has a potential to produce an artificial organ that can be implanted with the patient. However, the most difficulty in tissue reconstruction is the three-dimensional cell growth ability which could take very long period of time in healing process. This leading to an attempt to prepare a three-dimensional porous structure for cells seeding known as scaffold. It serves as a template for cell adhesion, proliferation, and differentiation into a specific tissue. Several requirements have been identified for the production of the scaffold for using in tissue engineering. The characteristics of an ideal scaffold [36] are

(i) Having interconnecting pores of appropriate scale to favor tissue integration and differentiation;

(ii) Making from appropriated material with controlled biodegradability or bioresorbability so that cells can differentiate into tissue and replace the scaffold;

(iii) Having appropriated surface chemistry to favor cells adhesion, proliferation and differentiation;

(iv) Having adequate mechanical properties to match the site of implantation and handling;

(v) Not induce any adverse response;

(vi) Easy to fabricate into a variety of shapes and sizes.

According to these requirements, many materials have been studied as the candidates to be adopted or synthesized and fabricated into scaffold. Naturally derived protein or carbohydrate polymers have been used as scaffolds for the growth of several tissue types. However, collagen is the most popular natural polymer used for tissue engineering. The examples of collagen membranes used as biomaterials for the application in tissue engineering were studied by Throm AM [43]. Mao C [29] studied the collagen hydrogels for functional cell-based biosensing. Morever, Khanna HJ [22] studied the applications of the composite of chitosan-collagen for hepatocyte implantation, Taguchi T [41] used collagen-calcium phosphate as an osteochondral scaffold. However, synthetic polymers are an attractive alternative in their application. The synthetic polymer in a group of aliphatic polyester such as polyglycolic acid (PGA), polylactic acid (PLLA), and poly(ε-caprolactone) (PCL) are commonly used as a biodegradable polymer to fabricate scaffold and many applications in bioengineering such as medical devices and drug delivery. They are also attractive materials to the growth of most tissues. There are many kinds of synthetic polymers using as biomaterials such as poly(L-lactic acid) (PLLA) [39]. There are many application of using polycaprolactone (PCL) [13,26,46]. The other polymer such as polypropylene and PTFE were studied by Danino AM [12] and polyurethane was used by Gulbins H [19]. The co-polymer such as polyglycolic acid (PGA)-polyglactin copolymer-polyhydroxyalkanoate (PHA) was studied by Shum-Tim D [38], poly(L-lactic-co-ε-caprolactone) [P(LLA-CL)] [45] and poly(ethylene glycol)-teraphthalate-poly(butylenes teraphthalate) (PEGT/PBT) [44] are the interesting alternative choices. A number of synthetic and natural polymers are used in many applications. However, only synthetic or natural polymer does not provide suitable properties for using in tissue engineering. Because almost of the synthetic polymers are limited to their bioregulatory activity due to the lack of bioactive functional groups, the natural polymer is always has a rapid degradation rate and

difficult to prepare. Blending of synthetic and natural polymer is an approach to develop the ideal biomaterials which exhibiting the combination of properties that could not be obtained by the individual polymers. Blending two polymers can provide the suitable of physicochemical properties such as an easy processing of synthetic polymer and the biocompatibility and biological interaction of natural polymer. The example of the synthetic and natural polymer composites are hydroxyapatite-collagen-PLLA [28], chitosan-γ PGA [20], chitosan-PCL [37], silk-PCL [9], and collagen-PCL [11].

Poly (ε-caprolactone) (PCL) is semi crystalline linear resorbable aliphatic polyester. It is thermoplastic resin made by polymerizing ε-caprolactone. The chemical structure of PCL is shown in Figure 2.1 and some properties of PCL are shown in Table 2.1.

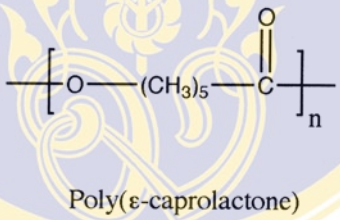


Figure 2.1. Chemical structure of poly (ε-caprolactone) [34]

On the other hand, PCL is a versatile synthetic polymer with low melting point which allows easy processing, low cost when compare to other biodegradable polyester. PCL also has good mechanical properties with compatible to soft and hard tissue. It has the susceptibility of its aliphatic ester linkage to hydrolysis. The products generated are either metabolized via the tricarboxylic acid (TCA) cycle or eliminated by direct renal secretion resulting in the lacking of toxicity [25].

Table 2.1 Properties of PCL (adapted from [1-2])

Properties	PCL
Inherent Viscosity (DL/G)	1.1 - 1.3
Freezing point (°C)	ca. 35
Melting Point (°C)	58 – 63
Glass Transition Temperature (°C)	-65 - -60
Decomposition Temperature (°C)	ca. 200
Solubility At 5% W/W	Dichloromethane, Chloroform, Hexafluoroisopropanol
Approximate Resorption Time (Months)	>24 (MW 80,000)
Specific Gravity (G/ML)	1.11
Tensile Strength (PSI)	3000 – 5000
Elongation (%)	300 – 500
Modulus (PSI)	$3 – 5 \times 10^4$

2.2 Scafffold preparations

To fabricate a good biodegradable scaffold for using in tissue engineering, many methods were studied to obtain the scaffold with appropriate properties such as highly porous of an appropriated scale with large surface/volume ratios to favor a large number of cells to attach and to allow the regeneration of the new tissue of the matrix. Several techniques have been developed to produce materials into porous structures such as solid freeform fabrication [36], Self-assembly [5], materials crosslinking [7,32], polymer extrusion [13], electrospinning [15,21,26] and solvent casting combined with particles leaching [9,35]. However, this research focused on electrospinning technique and solvent casting/salt leaching method.

2.2.1 Electrospinning technique

Electrospinning is a well-established process capable of producing ultra-fine fibers by electrically charging a suspended droplet of polymer melt or solution. There are many efforts to produce a polymer fiber into a nano range. Electrospinning is a well-known technique of producing fibers with diameter in a range of nano to a few microns. Recently, this technique has been developed and widely used in many applications since it has been introduced by Formhals in 1934 [26]. However, the use of electrospinning for biomaterials applications was reported by Martin and Cockshott as early as in 1977 [45]. In order to produce nanofiber from this technique, it uses electrostatic force for making fiber. A high voltage power supply is required to produce an electrically charged to draw a polymer solution from the tip of needle to a ground collector. As increasing the electric field, the semi-circular shape of hanging drop at the needle tip is charged a conical shape. It is known as the Taylor Cone [26]. The polymer jet is initiated when the electrostatic charge overcomes the surface tension of the drop. Nanofiber forms when the ejection of jet stream is traveled a spiral path to the ground collector and the evaporation of solvent. The parameters that affect morphology of electrospun fiber were reported by Lee KH [26]:

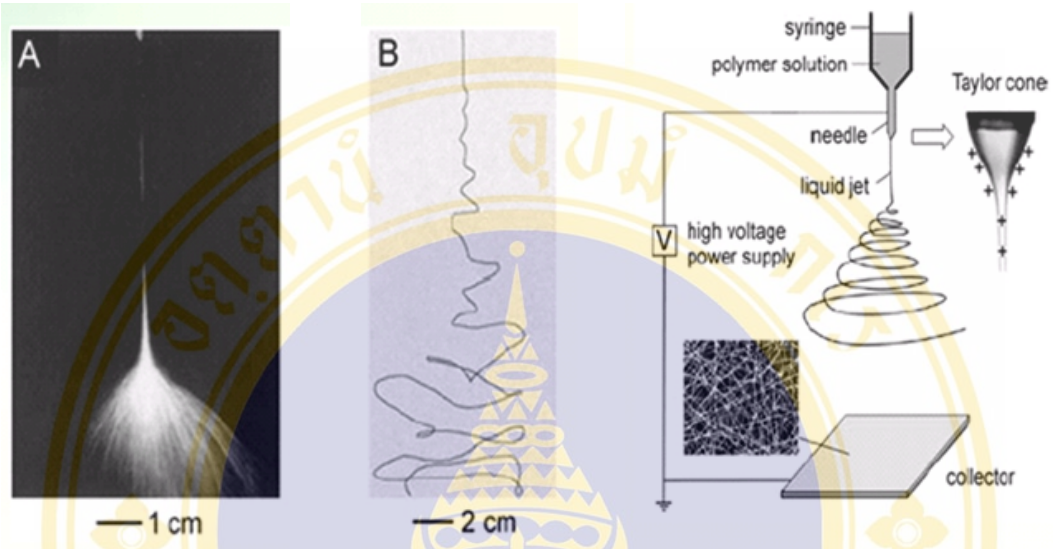
(i) solution parameters including viscosity, conductivity and surface tension;

(ii) controlled variables including hydrostatic pressure in the capillary, electric potential at the tip and the tip-to-collector distance;

(iii) ambient parameters including temperature, humidity and air velocity in the electrospinning chamber.

However, electrospinning is restricted to the complexity of the process and the factors to be considered. Viscosity of the polymer solution, electrospinning is restricted to its high viscosity. Dielectric constant and conductivity of solution is strongly affected on spinning and diameter of the fiber. As increasing dielectric constant, the diameter of fiber is decreased. The solvent composition and solution properties also play an important role in the fiber formation. There are the other parameter must be considered such as accelerating voltage, electrical intensity, injection rate and the distance between the needle tip and collector. The high accelerating voltage results in the high velocity of injection rate cause the decreasing

in fiber diameter. In opposite, the low accelerating voltage causes the dropping of solution.



(a) (b) (c)

Figure 2.2 Electrospinning process
(a) polymer jet, (b) a spiral path of polymer jet, (c) electrospinning system [27]

However, the beads have been often formed in electrospinning. This may cause from the instability of the jet of polymer solution. Moreover, the viscosity, net charge density and surface tension of solution are the parameter of bead forming [26]. Due to the small diameter of fiber and high surface area, electrospun fiber is beneficial for tissue engineering application. Moreover, the architecture is mimicked the natural tissue extracellular matrix (ECM) which benefit of improving mechanical properties and extensive for cell attachment when compare to larger fibers. Because of the complexity of the process, electrospinning is time consuming. The quantity of fiber generated is limited by the time constraints and the amount of polymer solution available. Moreover, the complex designed of scaffold is much more difficult than the other scaffold fabrication method.

2.2.2 Solvent casting/particles leaching technique

Solvent casting/particles leaching is one of the most common methods to fabricate porous scaffold. This method is the ease of fabrication without the need of specialized equipment. This method involves the leaching of a mineral salt usually NaCl or an organic compound such as saccharose [35] to generate the pores. The polymer solution is cast and the porogen particles are dispersed in a solution, then solvent is removed and follow by leaching out of the porogen. Due to casting and solvent evaporation step, this technique is suitable for thin scaffolds only. The major advantage of particles leaching method is the effective control of porosity and pore size. Scaffold with porosity levels up to 90% and pore size varying between 100 and 700 nm have been reported using this technique. With the addition of salt particles, the scaffold porosity can be tuned by modifying the salt concentration giving an additional degree of freedom in the scaffold design. Scaffold with the higher amount of porogen is clearly interconnected surface. The size of these pores varies throughout the scaffold and should depend on the size distribution of the salt particles. The pore morphology demonstrates good pore interconnection with the porous matrix. Also, the absence of remaining salt particles suggests that the dissolution of the salt crystals is completed and that the pores are well interconnected. The main advantage of this method is the ease of fabrication without the need of specialized equipment. The deficiency of this technique for the scaffolds with lower porosity levels is the lack of interconnected pores. Thus, decreasing the volume fraction of porogen particles decreases the amount of contact point between particles and also decreases interconnection between pores. Subsequently, this can lead to the entrapment of porogen in the polymer matrix. This occurrence is particularly important at low volume fraction of porogen, usually less than 65% for rigid spheres [35]. The limitation in the shapes (typically flat sheets and tubes are the only shapes that can be formed) and the possible retention of toxic solvent within the polymer. A few comments can be made on the pore morphology with respect to the tissue engineering application. The three dimensional regeneration of tissue by the aid of scaffold mostly depends on porosity, pore size, pore shape, pore distribution and overall shape of the object by means of the mechanical properties of the scaffold. Generally, a high porosity and a high interconnectivity are necessary to allow cell growth and penetrate

into scaffold. These pores also allow the transportation of nutrients and waste out of the scaffold. Another mention for scaffold is the size of interconnection between pores and size of pores. For salt leaching method, porosity is specified by the amount of leached particles, while the pore size and pore shape of the porous structure can be modified by varying the particles characteristics (size and shape). Even though the pore interconnection can be obtained using high porogen volume fraction, it cannot accurately be controlled. It clearly appears that the prepared scaffolds possess a good interconnectivity between the pores, and that is strongly desired in tissue engineering, as the exchange of nutrients and cell waste would be improved. However, it may be desirable to increase the size of the pore opening in order to improve the cell seeding throughout the whole sample and not only on its surface. Therefore, porosity is one of the parameter that must be considered.

2.3 Scaffold characterization

2.3.1 Hydrophobicity

According to the hydrophobic characteristic and lacking of biofunctional group that prevent the cell attachment onto the scaffold, blending organic substance to the polymer solution before preparing the scaffold helps to improve the hydrophilic characteristic of scaffold. This is an easy application of surface modifications, through the use of organic substance adsorption to provide more cues to cell attachment. The possibility of forming organic substance composites with biodegradable polymers are widely studied for example, PCL scaffold modified with silk [9], PCL thin films surface modification with acrylic acid and collagen [10], PCL scaffold blend with chitosan [37] and PCL/calcium phosphate scaffolds for tissue engineering [31]. This method helps to enhance the scaffold surface properties not only to promote cell adhesion and proliferation but also increase wettability of hydrophobic polymers and make suitable for using in tissue engineering.

The wettability can be determined in various situations and can be defined in term of water contact angle. The general observation is that the contact angle measured for a liquid advancing across a surface exceeds that of one receding from

the surface. It generally attributed to hydrophobicity and surface energy [42]. Although, the water contact angle can determine the hydrophobicity of the surface, the measurement of water contact angle is influenced by other factors i.e., surface roughness, surface heterogeneity, solution impurities adsorbing on the surface, swelling, and rearrangement or alteration of the surface by the solvent [3] resulting in the complexity of these parameters on the analysis. Therefore, the analysis must be carried out carefully. The decreasing in water contact angle is probably due to the increasing hydrophilicity of surfaces cause by adding the organic additive with hydrophilic characteristic. The effect of chemical composition on the contact angle will be determined by the competition between the non-polar and polar compositions on the surface. The differences of contact angle between these composite films are caused by the surface roughness from the particles. The attached particles on film surface caused the different inhomogeneous microstructure. Composite films which blend with the organic substances also have the lower contact angle than the pure PCL film. The increasing in surface roughness causes the decreasing in surface energy and the increasing in surface area, leading to the larger interaction between the water molecule and the film surface. The force balance between liquid-vapor surface tension of a liquid drop (γ_{sl}) and the interfacial tension between a solid and the liquid drop (γ_{lv}) can be evidenced through the contact angle (θ) of the liquid drop on the surface and can be used to characterize the surface energy (γ_{sv}). The basic relationship can be described in this form:

$$\gamma_{sv} = \gamma_{sl} + \gamma_{lv} \cos \theta \quad (1)$$

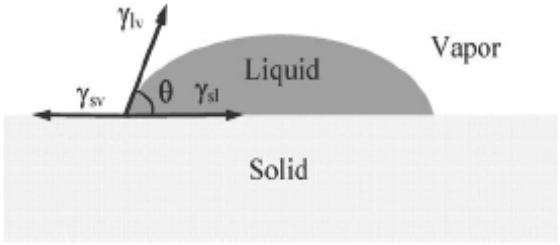


Fig. 2.3 Schematic of a sessile drop, contact angle,
and the three interfacial tensions [42]

The surface energy is directly related to surface wettability and correlated with biological interaction [34]. Nevertheless, the variations of contact angle on a surface depend not only on the chemical modification of the substances added but also on the morphological modifications of the modified surface. Contact angle is also affected by the surface roughness and it usually decreases with increasing surface roughness. The surface roughness may be caused from the density of particles attached on the film surface. Because of the non-uniform particle distribution, the shape of water droplet depended on its location on the surface. Conversely, contact angle is independent of the drop size [17]. According to Wenzel [47], the influence of roughness on the value of the contact angle has been quantified and it has the form:

$$r = \cos \theta_w / \cos \theta \quad (2)$$

where θ_w is Wenzel's angle (contact angle on a rough surface), θ is the contact angle on an ideally smooth surface, and r is the surface roughness. This means that contact angle usually decreases with the increasing surface roughness. Besides, the factors that affected the contact angle of each film surface are physiochemical properties of the surface included surface contamination, surface heterogeneity, reorientation or mobility of molecular segment at the surface, swelling, surface deformation [47], adsorption/desorption and interdiffusion of the water molecule on the surface.

2.3.2 Biodegradation

The effectiveness of scaffold depends on the designs and its properties for each application. Several characteristics of the scaffold such as water absorbed ability and degradation time should be considered before using in a practical cell culturing. The high absorption ability of the scaffold seems to be depended on the porous structure of the scaffold. The appropriated number of surface/volume ratios and the scale of the pores are the parameters which show a good absorbent characteristic of the scaffold. The water uptake percentage can be calculated from the content of the distilled water in the absorbent scaffolds. Contact angle can use to define the wettability of the surface and also refer to the porous scaffold properties. The %water uptake of the scaffold is measured to study the relationship between the decreased

contact angle and the ability to absorb water of the sample. Since the scaffolds are ready porous, the volume of the scaffold does not affect the water absorption ability significantly.

Whatever application of PCL scaffold, most are related to tissue engineering. The requirements for materials to use as a substrate for the development of cells are not only related to the correct adhesion and proliferation but also to the mechanical characteristic because their useful lifetime, maintenance and replacement.. Since these substrates ensure the appropriate performance until the new tissue is capable of restoring the original function. Therefore, biodegradable polymer with desired degradation rates should be synthesized and the degradation rate should be determined by an effective method. Generally, degradation mechanism of the PCL molecules is affected by their chemical structure and structural characteristics [33]. The degradation mechanism is governed by autocatalysis due to carboxylic end groups. The degradation of PCL carries on two stages: random hydrolytic ester cleavage and weight loss through the diffusion of oligometric species from the polymer [33]. The degradation rate is enhanced by both hydrophobic/hydrophilic balance and crystallinity. The PCL degradation mechanism was known as random hydrolytic chain scission of the ester linkage [25]. Enzymes may be defined as a catalyst of the interaction. This is more evidence to demonstrate that esterase and lipase enzymes can accelerate the degradation rate of PCL. And it also proved the biodegradability of PCL and the specific enzymatic action of the lipase [8].

2.3.3 Residual solvent

One of the problems during solvent casting/salt leaching method is the presence of organic solvent which may be entrapped in the scaffold during drying process. This could cause a toxicity to the cell during culturing process. This can be monitored easily using FTIR to determine the residual chloroform or other solvents presenting in the sample. This infrared (IR) spectrum will show the absorption of IR spectra due to the presence of chemical functional groups in the sample via bending, vibrating, or stretching absorption energies. Fourier transform-infrared spectroscopy (FT-IR) is well established as an analytical technique for functional group analysis

and to study the hydrogen bonding and phase separation behaviour in polymers, since mid-infrared spectral changes in band intensity and frequency shifts are known criteria for the presence and strength of hydrogen bonds. In general polymer or macromolecules possess bonds and functional groups characteristics of their identity. These functional groups vibrate independently of each other and weakly interact. It is well known that the total energy of a particular bond or functional group in a macromolecule arises from the contribution of translational, rotational, vibrational, and electronic energies [30]. Therefore, an interaction with radiation of the electromagnetic (EM) spectrum will result in different energy transitions of the bond or functional group involved in the macromolecule. Without any EM radiation effect, these bonds or functional groups vibrate independently at their equilibrium position. The local vibration depends on the intermolecular interactions such as weak London forces or hydrogen bonding phenomenon if any [30]. Therefore, the equilibrium vibration of a bond or functional group depends on the local geometry involved. However, when IR radiation (which is part of the EM spectrum) is applied to a functional group, it breaks down the equilibrium (position) stage, causing two energy transitions. It promotes transitions in a macromolecule between rotational and vibrational energy. When transitions between rotational and vibrational energy levels occur and cause a net change in the dipole moment, the molecule will absorb IR. Therefore, an IR absorption profile is unique to a specific molecular vibration frequency. When IR radiation passes through a sample, some of it is absorbed by the sample and some of it is passed through (transmitted). The resulting spectrum represents the molecular absorption/transmission, which creates a fingerprint of the analyzed sample. Therefore, identification of functional groups is a major application of IR spectrometry. Thus, it should be realized that any change in a peak position or shape means a change has occurred in the distribution of frequencies included in that particular vibration mode. Nevertheless, Mishra AK [30] suggested that the complicacy of analytical the structure–property correlation in a polymer with spectral deconvolution arises great scientific challenges due to:

(i) spectral investigation of hydrogen bonding is complicated not only by multiple overlapping peaks but also by other band distortions effects;

(ii) a proper baseline correction is needed;

(iii) the time during the scanning should be high, so that the effect of noise can be minimized;

(iv) care should be taken not to force fit the bands, which may generate erroneous results, etc.

The commonly band for determination of chloroform is a strong absorption band at 761.7 cm^{-1} corresponds to the C-Cl functional group as showed in Table 2.2. According to Elzein T [16], a characteristic absorption band for PCL is at 1727 cm^{-1} corresponding to the carbonyl stretching functional group as showed in Table 2.3.

Table 2.2 Characteristic infrared bands of chloroform

Position (cm^{-1})	Vibrator
3020	C-H stretching
1214.9	C-H bending
761.7	C-Cl stretching

Table 2.3 Characteristic infrared bands of PCL [16]

Position (cm^{-1})	Vibrator
2949	Asymmetric CH_2 stretching
2865	Symmetric CH_2 stretching
1727	Carbonyl stretching
1393	C-O and C-C stretching in the crystalline phase
1240	Asymmetric COC stretching
1190	OC-O stretching
1170	Symmetric COC stretching
1157	C-O and C-C stretching in the amorphous phase

By comparing the absorption bands of chloroform in table 2.2 versus that of PCL shown in table 2.3, it is clearly seen that the easiest method to determine the residual chloroform that may present in the samples by monitoring the residual C-Cl stretching band in all samples.

CHAPTER III
MATERIALS AND METHODS

This research fabricates the scaffold, performs the properties testing and analyses the results in order to make an understanding regarding the possibility of using the scaffold for tissue engineering. This research used methods and techniques as follows:

3.1 Materials used in this research

In this research, materials and chemicals are selected to be used to prepare the samples and perform the experiment. The instrument was used to determine the interesting properties of the scaffold.

3.1.1 Materials and chemicals used in this research are as following:

1. Polycaprolactone (PCL) powder with molecular weight 80,000 was obtained from Solvay caprolactones, Warrington, England.
2. Chloroform, acetone and dichloromethane (DCM) analyze grade was used.
3. Organic substances such as collagen, chitosan, chondroitin sulfate, gelatin and glucosamine HCL are purchased from Sigma-Aldrich, Inc., St Louis, MO, USA.
4. Sodium Chloride (NaCl) with the average particle size 100-300 μm.
5. Lipase type II crude from Porcine Pancreas was purchased from Sigma-Aldrich, Inc., St Louis, MO, USA.
6. Distilled water
7. Phosphate buffer solution 0.02 M
8. Vitros Chemistry Products LAC Slides

3.1.2 Instruments used in this research are as following:

1. Scanning Electron Microscope (SEM) (Philip: XL30&EDAX)
2. Contact angle goniometer (Krüss GmbH, Hamburg, Germany: G-23)
3. Scanning probe microscope controller (Digital instrument Veeco Metrology Group:Nanoscope IIIa)
4. Electrospinning system: high voltage generator (RHVS100-1000 N/T) and syringe pump (TERUMO:TE-331)
5. Balance (Denver Instrument Company:TC-254)
6. FTIR spectrophotometer
7. Vitros Chemistry System (Vitros 250)

3.2 Methods

This research studies about PCL scaffold fabrication to obtain the PCL porous structure using electrospinning technique and solvent casting/salt leaching technique. The obtained scaffold was applied to the cell culturing study for observing the possibility to use it in tissue engineering.

3.2.1 Study of scaffold fabrication

The effect of solvent to PCL scaffold fabrication

In this study, PCL powder was dissolved in the specific solvents that are chloroform, acetone and dichloromethane (DCM) at the concentration of 5% and 10% w/v. The solutions are stirred using magnetic stirrer to enhance the miscibility and then the solution was cast onto the glass, leave air-dried at room temperature about 1 day. After that PCL films are removed from the glass and kept in the desiccator.

Scaffold fabrication using electrospinning technique

Electrospinning is a technique used to produce the PCL fiber with diameter in the range of nano to micron using electrically driven jet of polymer solution. In this study, the electrospinning system was composed of three important parts that are high voltage generator, syringe pump and ground electrode which is used to collect the produced nanofiber. The experimental devices are set up as shown in figure 3.1.

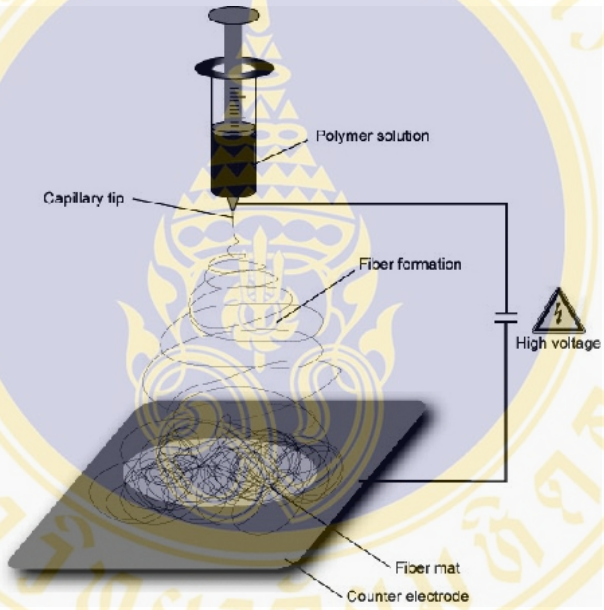


Figure 3.1 Experimental set up devices for electrospinning

The voltage used was ranging from 10-50 kV. The electricity was ranging from 0.1-0.5 mA. PCL powder was dissolved in two solvent systems that are dichloromethane (DCM) and the mixture between DCM and dimethyl formamide (DMF). The concentration of the solution is 8% w/v. PCL nanofiber was produced from the mixed solution of PCL in DCM and DMF at the ratio 3:1.

Scaffold fabrication using solvent casting/salt leaching technique

PCL powder was dissolved in chloroform at the concentration 10% w/v. The solution was stirred with magnetic stirrer to enhance the miscibility. Sodium chloride (NaCl) was blended to PCL solution as a porogen. Particle size of NaCl used was in the range of 100-300 μm. The quantity of NaCl used was 1:10 and 1:25 weight ratios of PCL:NaCl. The mixture was cast onto Petri dish and leave air-dried at room temperature about 1 day. Then PCL scaffold was soaked in distilled water for 2 day to remove salt from the scaffold. The distilled water was renewed everyday. Afterthat, the scaffold was removed from distilled water, left air-dried at room temperature until dry and kept in the desiccator. The procedures are shown in figure 3.2.

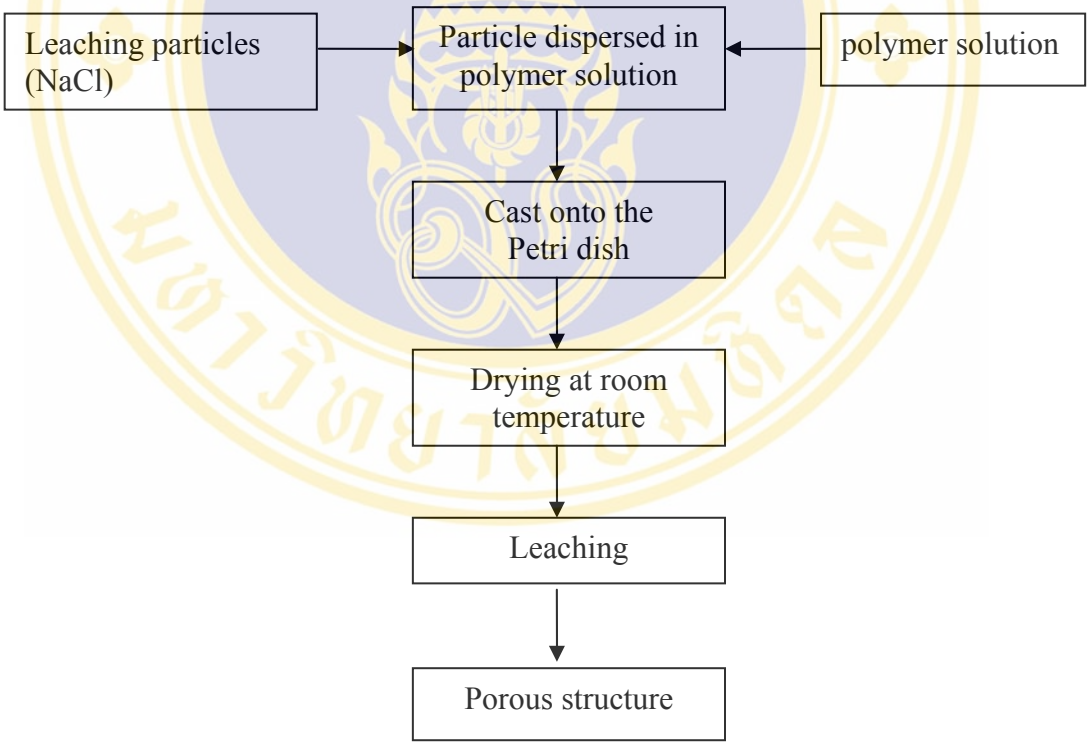


Figure 3.2 Procedure of scaffold fabrication by salt leaching technique.

3.2.2 The PCL scaffold properties

According to the fabrication of PCL scaffold with electrospinning and solvent casting/salt leaching technique, the obtained scaffolds show the hydrophobic character due to polymer property. However, this limitation can be improved by blending organic substance to PCL solution before fabricated into scaffold.

In this study, the organic substances that are collagen, chitosan, chondroitin sulfate, glucosamine HCL and gelatin are added to the PCL solution respectively at weight ratios of organic powder:PCL 1:1. The solution then fabricates into a PCL composite film and study for its surface properties. However, roughness of surface may affect the wettability of the film. So, this study also blends the organic solution to PCL solution in order to reduce the influence of roughness from organic particle to the wettability of the film. The PCL composite film was prepared using as described before. But the PCL solution was blended with organic solution instead of powder. The concentration of the organic solution was 1% w/v. The ratio of the mixture was PCL solution:organic solution 10:1.

Afterthat, the PCL film will be tested for wettability and roughness. The wettability of films was assessed by optical water contact angle measurement using contact angle goniometer. The roughness of film surface was examined using atomic force microscopy (AFM) by the aid of scanning probe microscope controller.

For the practical used, scaffolds must be prepared into a porous structure. This study fabricates PCL scaffold using solvent casting/salt leaching technique and blend with organic substances as describes. And they will be studied for morphology using scanning electron microscope (SEM), water absorption test, the enzymatic degradation rate and the residual chloroform in the sample using FTIR spectra.

The swelling test can be performed as briefly describe. The PCL composite scaffold with the size about 1 cm. x 1 cm. was weighed for the dry weight. The 3 ml. of distilled water was added to PCL sample and soaked at room temperature for 5 minutes. After removed the exceed water using pipette, they are weighed again for the wet weight. The content of distilled water in the absorbed membrane was calculated according to the equation for %water uptake analysis by Park SN [32] as shown below:

$$\text{Water uptake (\%)} = \frac{(Ws - Wd)}{Ws} \times 100,$$

where Wd is the weight of the dry scaffold

Ws is the weight of the swollen scaffold.

The enzymatic degradation test of PCL scaffold was carried out at 37 °C in lipase solution (pH 7.4). The PCL scaffolds with the size about 1x1 cm. are weighed for the initial weight (W_b) and placed into the Petri dish. Then 3 ml. of lipase solution was added to the sample. The components of the lipase solution are 1 mg lipase/ml of PBS. The medium was renewed every 3 days. The samples are picked up periodically at day 1, 3, 7, 11 and 15 of the test then washed with distilled water followed with 70% ethanol and left air-dried in fume hood at room temperature. The dry samples are weighed for a constant weight (W_a). The enzymatic degradation was determined as percentage weight loss and calculated according to the equation for %weight loss analysis by Chen DR [8], Darwis D [14], and Gan ZH [18], as shown below:

$$\text{Weight loss (\%)} = \frac{(Wb - Wa)}{Wb} \times 100,$$

where Wb is the weight of the initial scaffold before enzymatic test

Wa is the weight of the scaffold after enzymatic test.

Infrared absorption spectra of PCL film are obtained from FTIR spectrophotometer. The FTIR measurement was performed over the range of 370-4000 cm^{-1} with 100 scans. PCL films are prepared as described before but the picked up period are 1, 3 and 5 days to observe the C-Cl band spectrum of residual chloroform in the PCL film. The result of this study is to ensure that there is no residual chloroform in the sample when using in the cell culturing.

3.2.3 Study of possibility of using in tissue engineering

Scaffold for practical cell culture using will be studied for the possibility of using in tissue engineering by hepatocyte cell culturing *in vitro*.

Finally, hepatocyte cells are cultured onto the scaffold.The scaffolds are cut into 1x1 cm. and soaked in 70% alcohol for 5 minutes. After that samples are washed and dialysis with 70% alcohol 2 times. The wet scaffolds are washed with rpmi media to remove the residual alcohol. The samples are kept in Petri dish. The sterilized scaffolds are wet with rpmi media and placed on the Petri dish (diameter 3.5 cm.), 1 piece per Petri dish. The excess media was gradually removed with pipet. Hepatocyte cells are seed to each sample with the cell density about $5x10^4$ cells per sample and incubated at 37 °C and 5% CO_2 for 30 mins to let the cell attached onto the scaffold. After 30 minutes of incubation, 1 ml. of media was added to scaffold. Cells are cultured for 7, 14 and 21 days. The media was renewed every 3 days. The samples of each scaffold type are seeded in triplicate to obtain each time point for studying of cell morphology and cell viability. The cell morphology was observed using SEM and cell viability was determine the quantity of lactic acid from the cell metabolism by the aid of Vitros Chemistry System.

3.2.4 Statistical analysis

The data were subject to statistical analysis using an ANOVA using MATLAB version 7.0. Significant differences were reported for $P \leq 0.05$.

CHAPTER IV
RESULTS

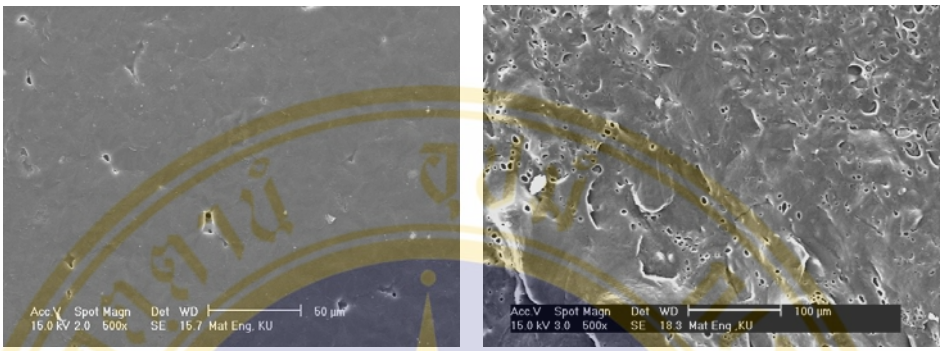
4.1 PCL scaffold fabrication

The effects of solvent to PCL scaffold fabrication were performed on various solvent. The results of PCL powder dissolutions in various solvent systems were shown in Table 4.1.

Table 4.1 The dissolution of PCL powder in three solvent systems at the concentrations of 5 and 10%.

Solvent	Results	
	5%	10%
Chloroform	Good dissolution	Moderate dissolution
Dicloromethane	Moderate dissolution	Slow dissolution
Acetone	Slow dissolution	Partial dissolution

The SEM micrographs of solution cast PCL film were shown in figure 4.1.



(a) (b)

Figure 4.1 Effect of concentration on the surface morphology of solution cast PCL film, (a) 5% in chloroform, (b) 10% in chloroform

Scaffold fabrication using electrospinning technique

Electrospun PCL nanofibers were prepared in condition as shown in Table 4.2.

Table 4.2 The optimum conditions for electrospinning in various solvent.

solvent	Injection rate (ml/h)	Potential (kV)	Distance between needle tip and collector (cm.)
DCM	0.8	6.5	12
DCM	1.1	8.5	12
DCM:DMF (3:1)	1.0	11	15

The SEM micrographs of electrospun PCL fiber were shown in figure 4.2.

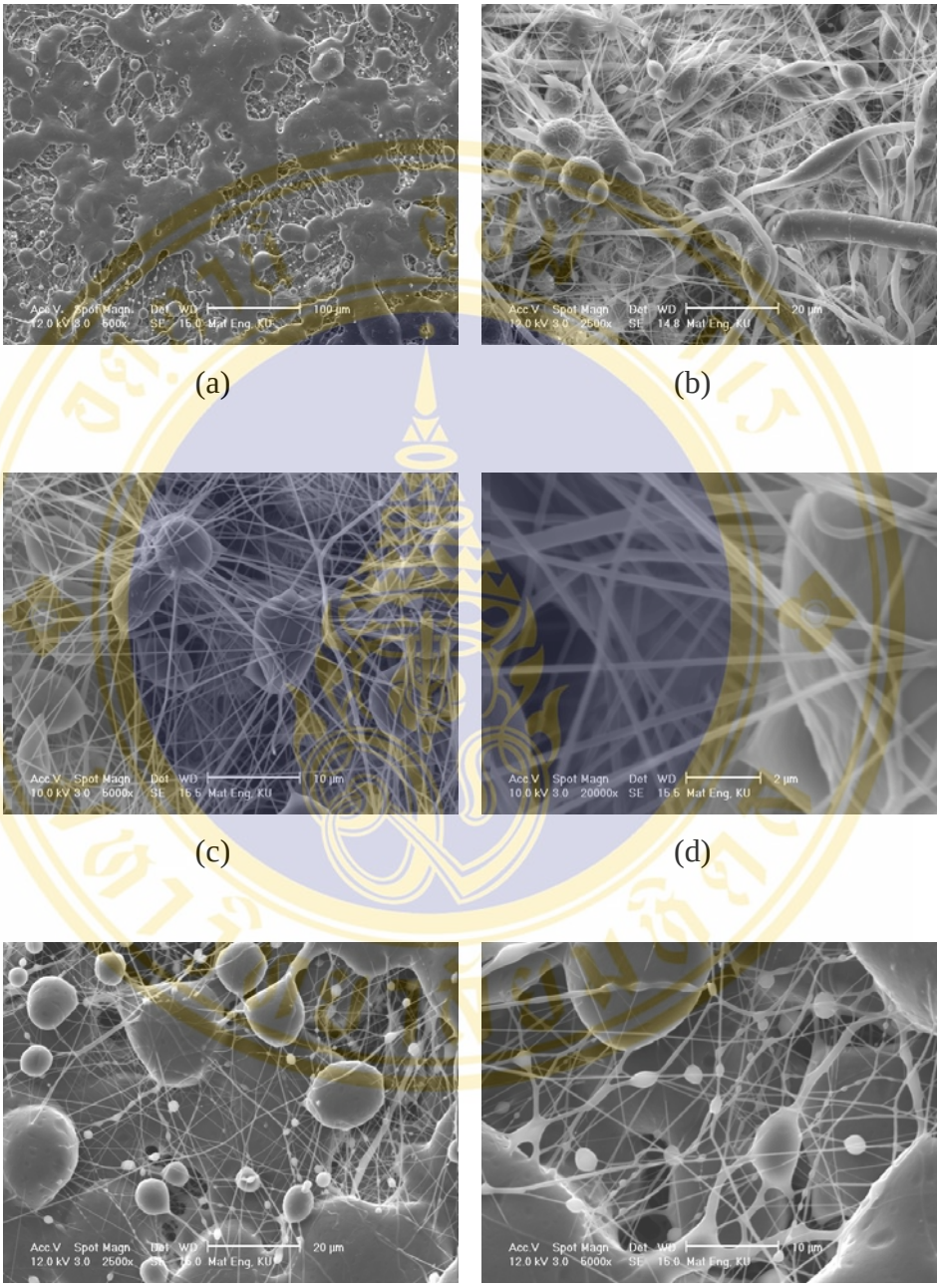
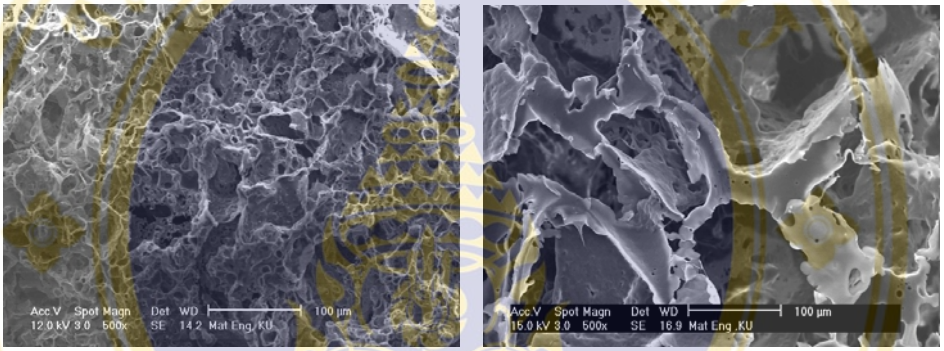


Figure 4.2 SEM photographs of PCL fiber from three conditions.
(a)-(b) DCM, injection rate 0.8 ml/h at 6.5 kV, d= 12 cm.,
(c)-(d) DCM:DMF (3:1), injection rate 1.0 ml/h at 11 kV, d= 15 cm.,
(e)-(f) DCM, injection rate 1.1 ml/h at 8.5 kV, d= 12 cm.

Scaffold fabrication using solvent casting/salt leaching method

A variety of techniques are available for porous scaffold fabrication, including solvent casting/salt leaching. This method provides easy control of the pore structure and has been well established.

The SEM micrographs of PCL scaffold with different ratios of NaCl particles are shown in figure 4.3(a) and (b).



(a) (b)

Figure 4.3 SEM photographs of scaffold after extraction of NaCl at different weight ratio
(a) 1:10, (b) 1:25

4.2The PCL scaffold properties

To improve surface hydrophilicity, the organic substances: collagen, chitosan, chondroitin sulfate, glucosamine HCL and gelatin were added to the PCL solution respectively in the mass ratios of organic powder:PCL 1:1. The PCL composite solution was cast into a film. The morphology of the composite PCL film prepared using solvent casting method was investigated using SEM to identify any changes due to the organic substance added. The results were shown in figure 4.4.

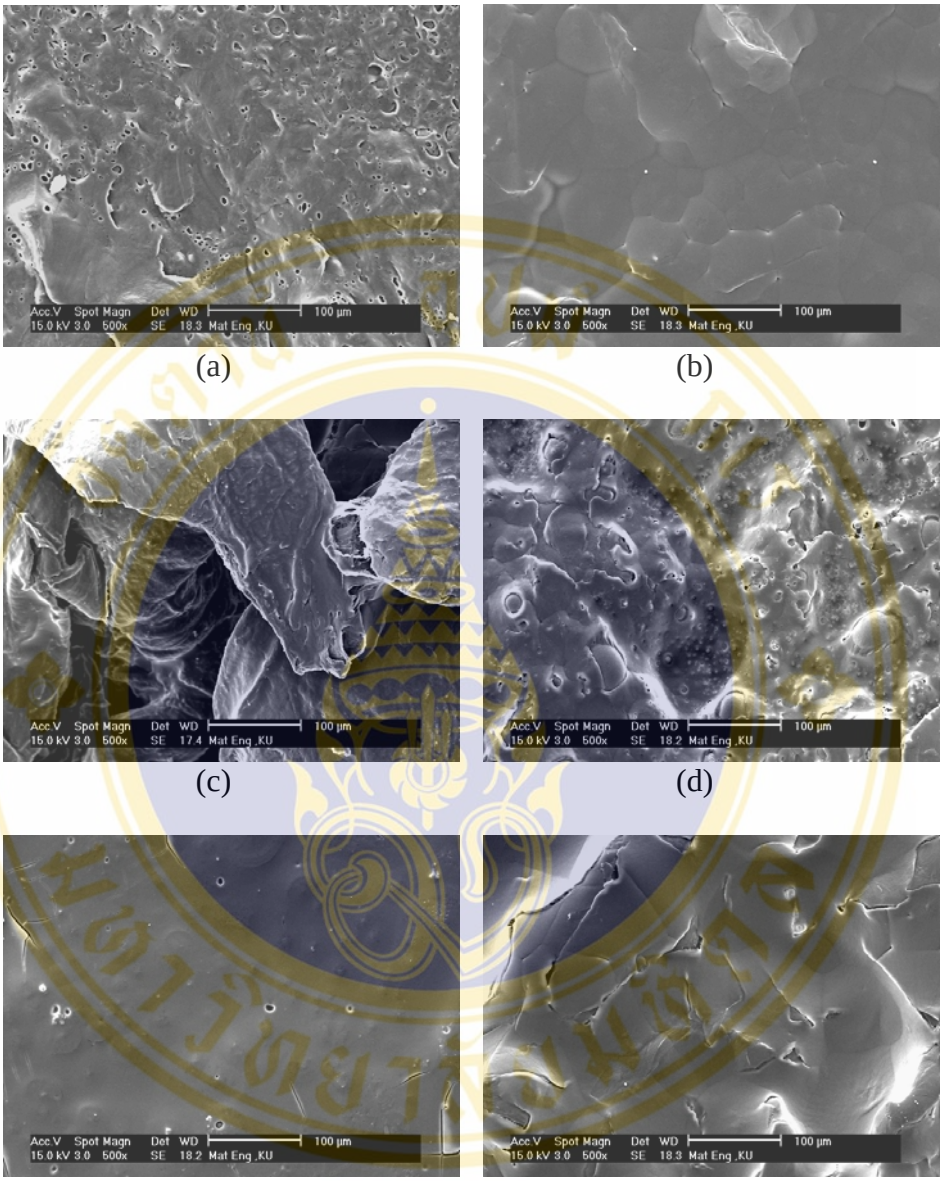


Figure 4.4 SEM photographs of PCL composite film blended with organic powder. (a) PCL film, (b) PCL-glucosamine HCL, (c) PCL-chitosan, (d) PCL-collagen, (e) PCL-chondroitin sulfate, and (f) PCL-gelatin.

The wettability is assessed by optical water contact angle measurement (advancing contact angle) whose results were shown in Table 4.3.

Table 4.3 The advancing contact angle on the PCL composite film surface as a function of organic powder added.

Sample	Contact angle (°)	SD
PCL film	99.39	1.84
PCL:chitosan (1:1)	N/A*	N/A*
PCL:chondroitin sulfate (1:1)	59.50	5.72
PCL:gelatin (1:1)	61.73	7.51
PCL:glucosamine HCl (1:1)	56.80	9.96
PCL:collagen (1:1)	20.38	2.32

* Not applicable

.

Since the contact angle measurement is not only affected by the hydrophobicity of the surface but also related to the surface roughness of the samples. Therefore, the measurement of surface roughness is also carried out and the results were given in Table 4.4.

Table 4.4 Roughness (Ra) of PCL composite film blended with organic powder

Sample	Surface roughness (nm)	SD
PCL film	79.035	0.68
PCL:chitosan (1:1)	N/A*	N/A*
PCL:chondroitin sulfate (1:1)	163.99	0.88
PCL:gelatin (1:1)	278.84	1.51
PCL:glucosamine HCl (1:1)	325.13	0.78
PCL:collagen (1:1)	386.82	0.73

* Not applicable

In order to eliminate the effect of surface roughness on the water contact angle measurement, the organic additives were dissolved before adding in the prepared polymer solutions and the contact angle measurement were carried out and the results were given in Table 4.5.

Table 4.5 The advancing contact angle on the PCL composite film surface as a function of organic solution added.

Sample	Contact angle (°)	SD
PCL film	99.39	0.35
PCL:chitosan (1:1)	96.70	4.76
PCL:chondroitin sulfate (1:1)	92.00	10.54
PCL:gelatin (1:1)	98.91	9.67
PCL:glucosamine HCl (1:1)	96.73	4.73
PCL:collagen (1:1)	70.55	5.82

The measurement of surface roughness is also carried out on the PCL composite film mixed with organic solution and the results were given in Table 4.6.

Table 4.6 Roughness (Ra) of PCL composite film blended with organic solution.

Sample	Surface roughness (nm)	SD
PCL film	79.035	0.68
PCL:chitosan	84.272	0.57
PCL:chondroitin sulfate	82.520	0.84
PCL:gelatin	80.080	0.92
PCL:glucosamine HCl	83.566	2.42
PCL:collagen	87.074	0.617

The morphology of the composite PCL scaffold prepared using solvent casting/salt leaching method was investigated using SEM to identify any changes due to the organic substance added. The results were shown in figure 4.5 and 4.6.

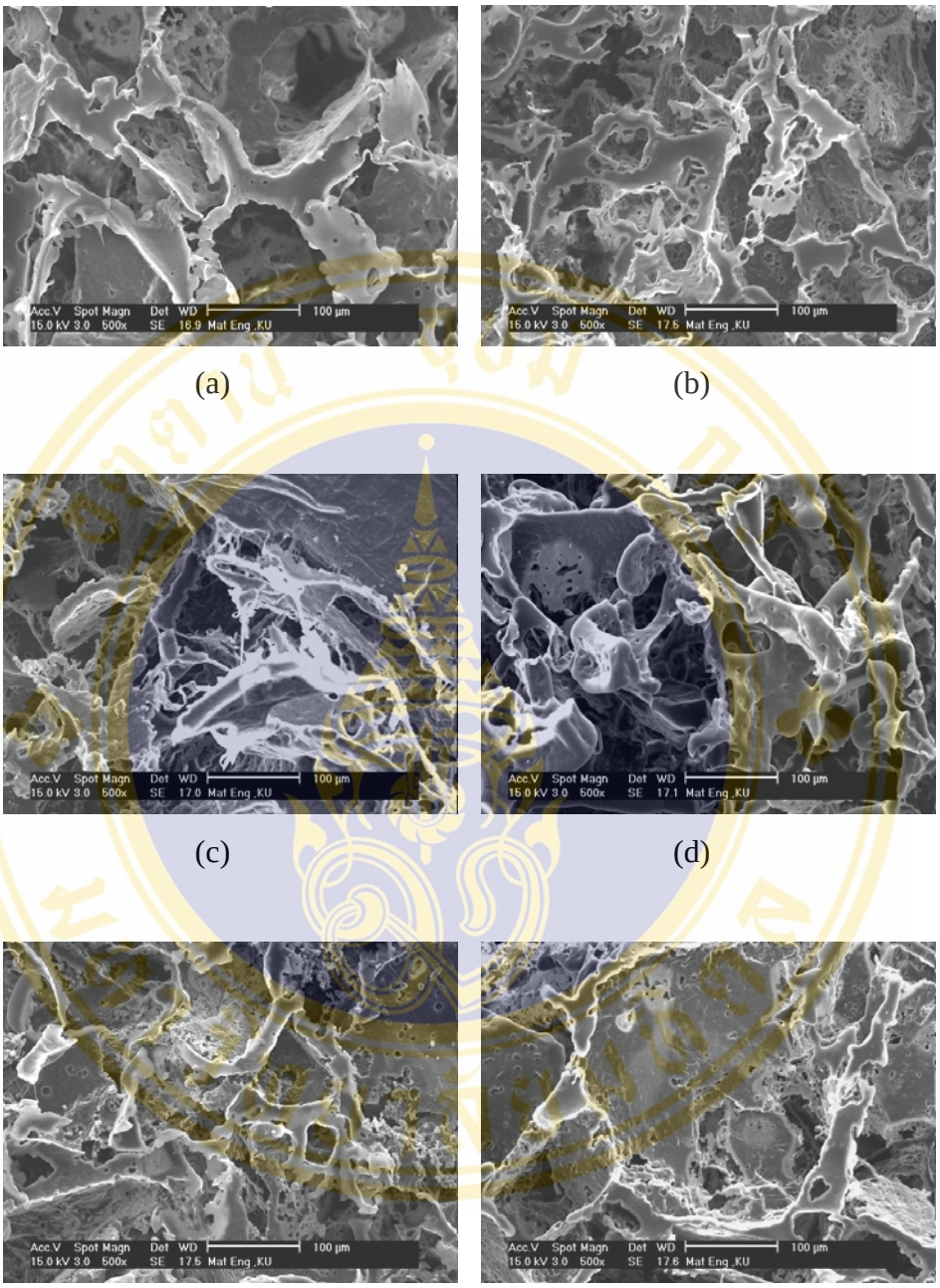


Figure 4.5 SEM photograph of PCL composite scaffold blended with organic powder. (a) PCL scaffold, (b) PCL-glucosamine HCL, (c) PCL-chitosan, (d) PCL-collagen, (e) PCL-chondroitin sulfate, and (f) PCL-gelatin.

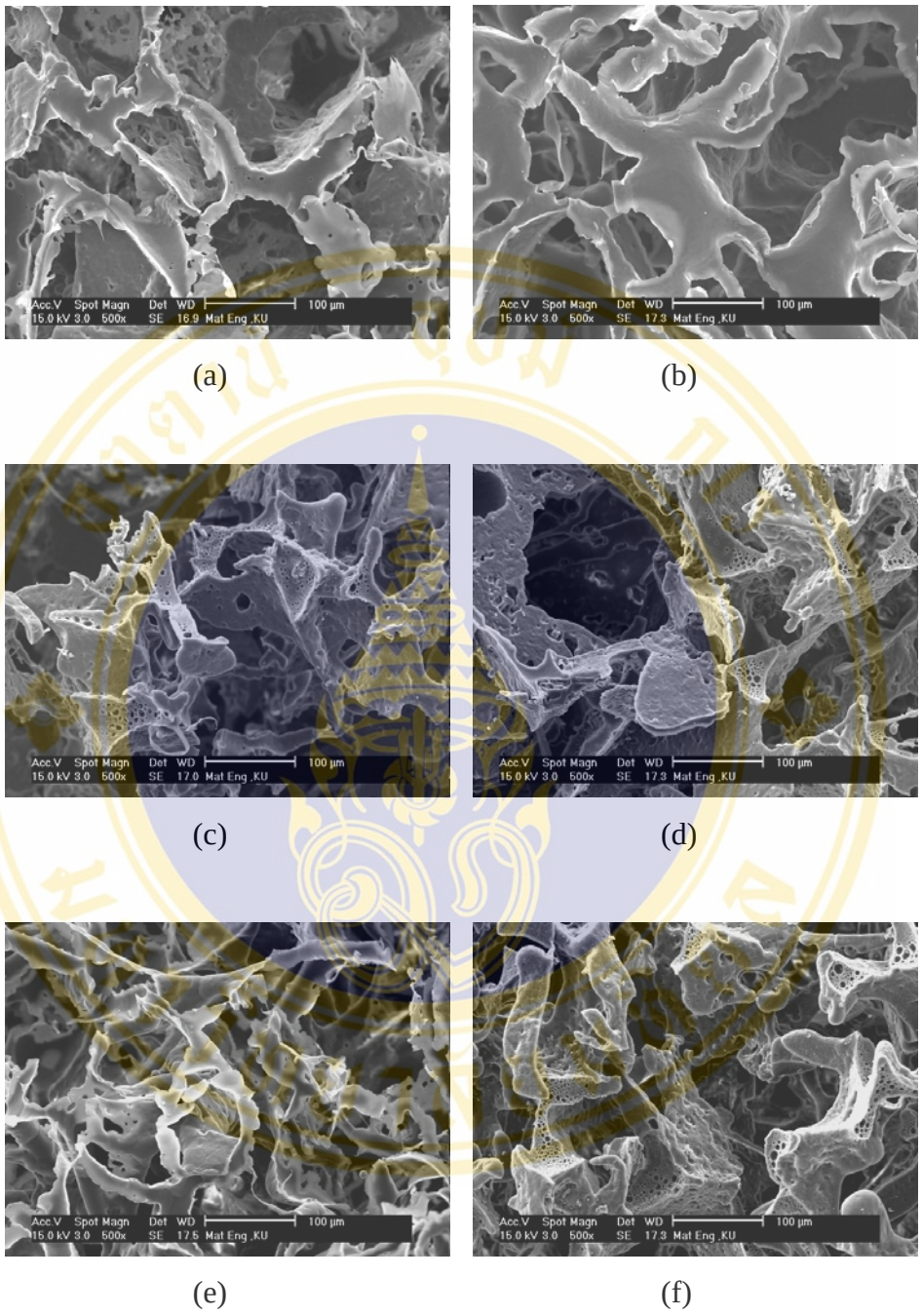


Figure 4.6 SEM photograph of PCL composite scaffold mixed with organic solution. (a) PCL scaffold, (b) PCL-glucosamine HCL, (c) PCL-chitosan, (d) PCL-collagen, (e) PCL-chondroitin sulfate, and (f) PCL-gelatin.

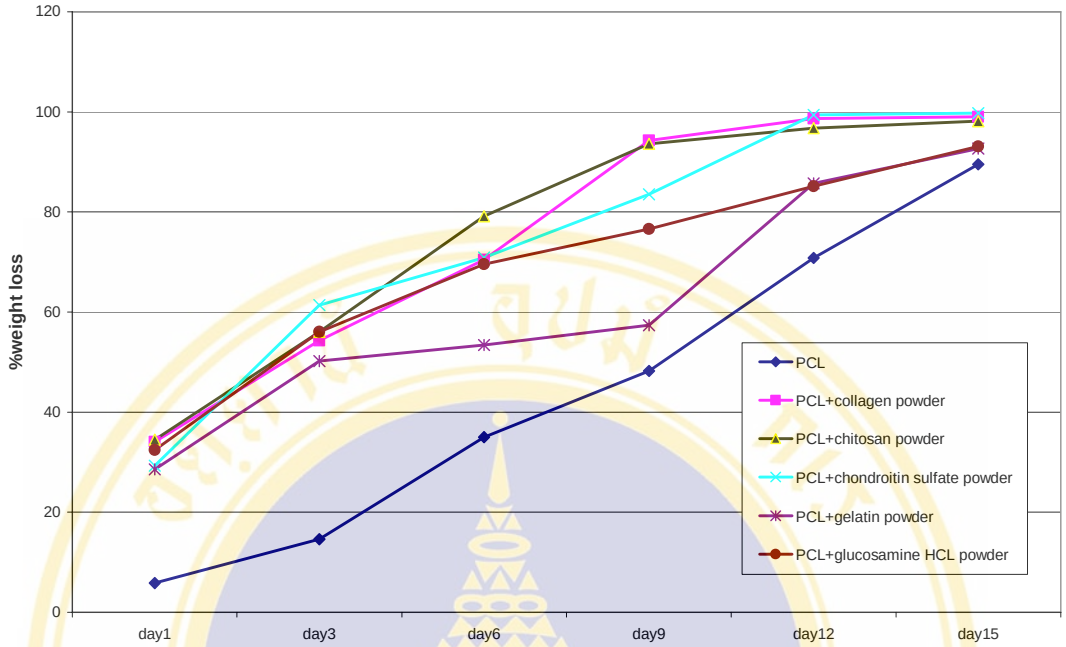
To evaluate the quality of the prepared sample for scaffold using, the water absorption on each sample was conducted. The results were shown in Table 4.7.

Table 4.7 % water uptake of PCL composite scaffold

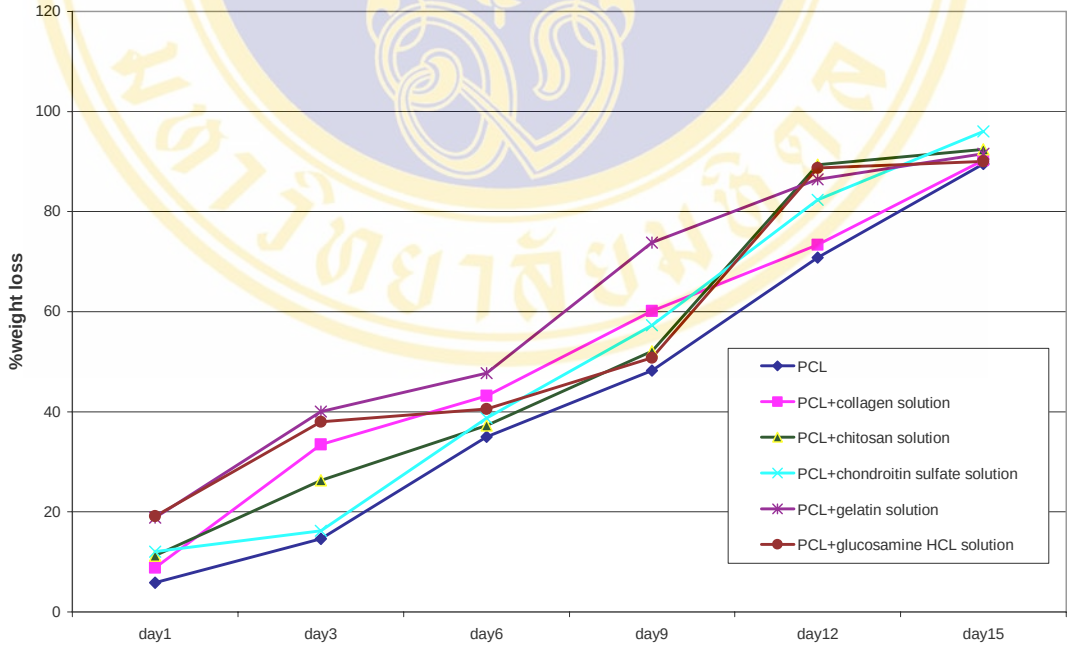
Sample	% water uptake	
	Organic powder	Organic solution
PCL scaffold	86.04	
PCL:chitosan (1:1)	88.17	92.54
PCL:chondroitin sulfate (1:1)	81.20	90.42
PCL:gelatin (1:1)	88.87	92.50
PCL:glucosamine HCl (1:1)	79.53	90.25
PCL:collagen (1:1)	89.86	93.76

The study also composed of the enzymatic degradation testing on prepared samples and the determination of residual solvent in sample to evaluate the proper solvent evaporation time for scaffold preparation

The different scaffolds were compared the stability to the enzymatic degradation using % weight loss after being soaked with lipase solution. Effect of adding organic powder and organic solution on the enzymatic degradation of the PCL composite scaffold was shown in figure 4.7(a) and (b), respectively.



(a)



(b)

Figure 4.7 %Weight loss of the scaffold during the degradation test.
(a) scaffold blended with organic powder,
(b) scaffold mixed with organic solution.

Evaporation of the solvent deposited on scaffold surface was studied with FTIR spectroscopy to observe the C-Cl band spectrum of residual chloroform in the PCL film. Measurements were taken at day 1 and 3 of solvent evaporation. The results spectra were compared to the PCL melt casting film spectrum as background.

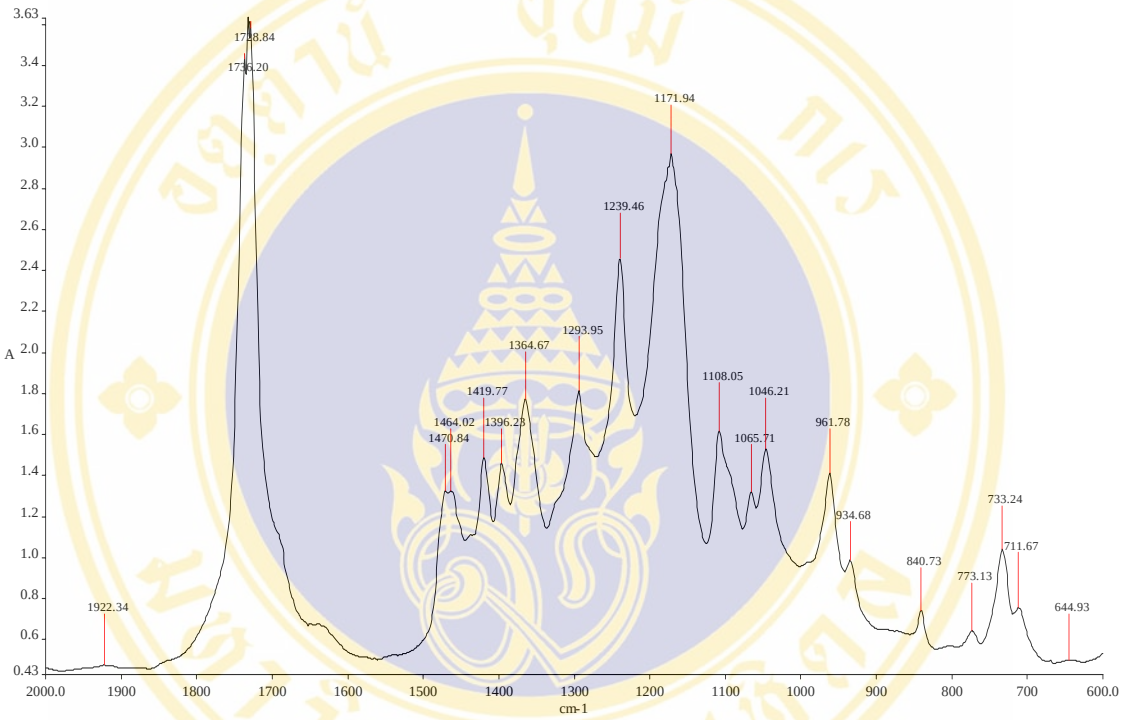


Figure 4.8 FTIR spectrum of PCL melt casting film

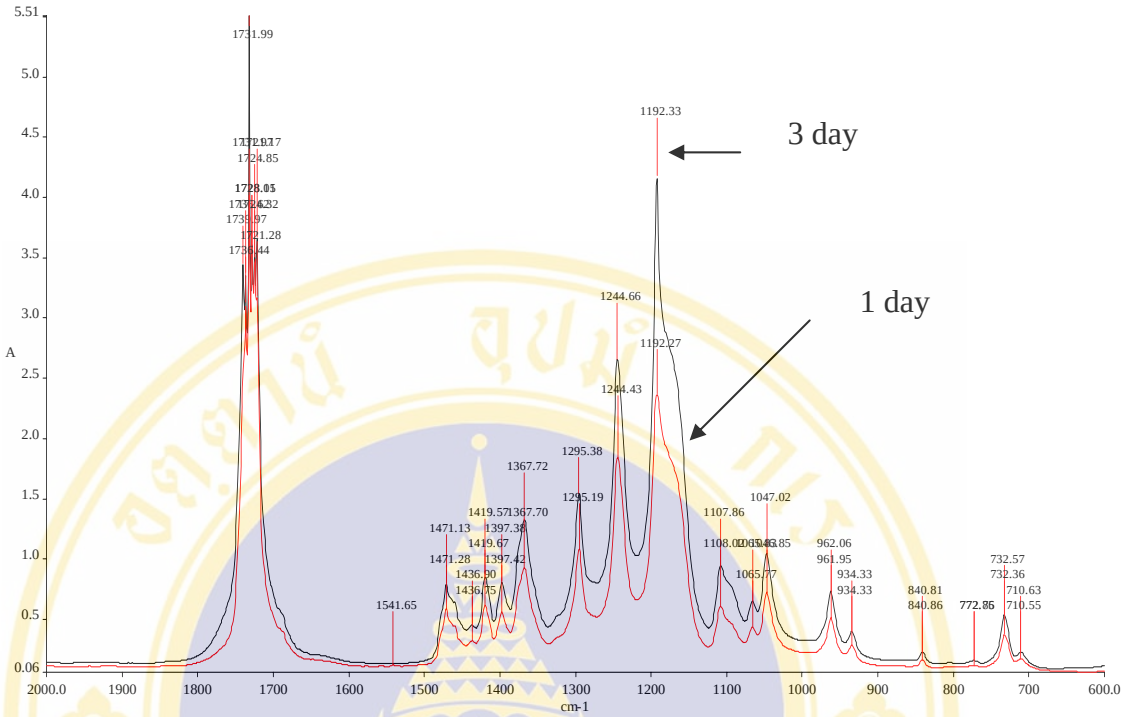


Figure 4.9 FTIR spectrum of the evaporated PCL film taken at 1 and 3 days.

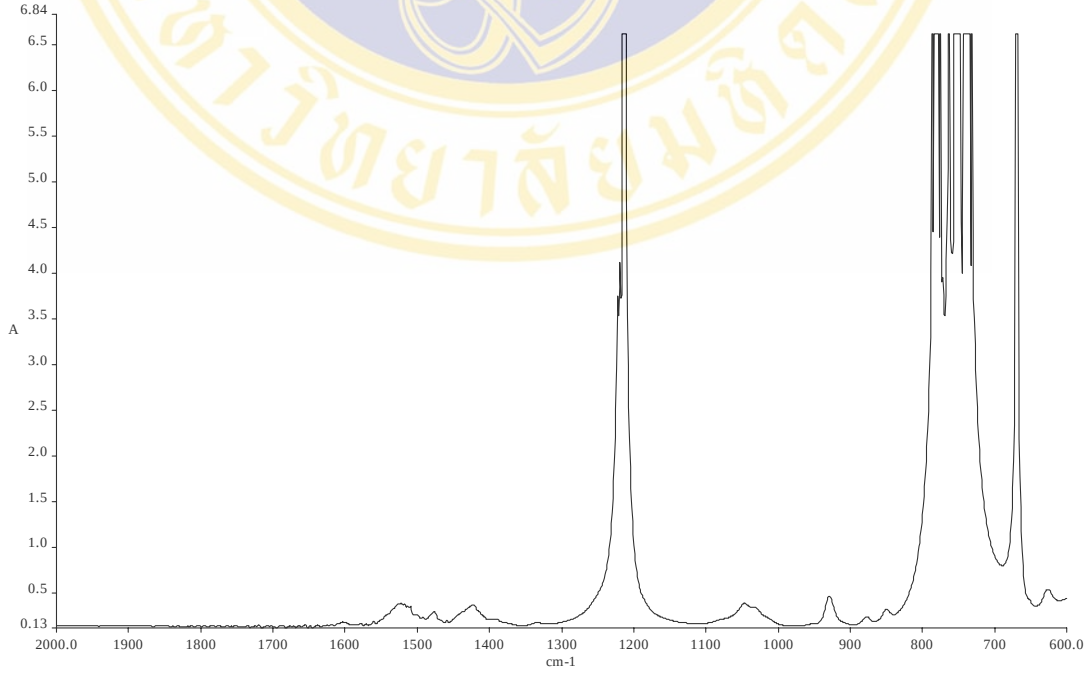
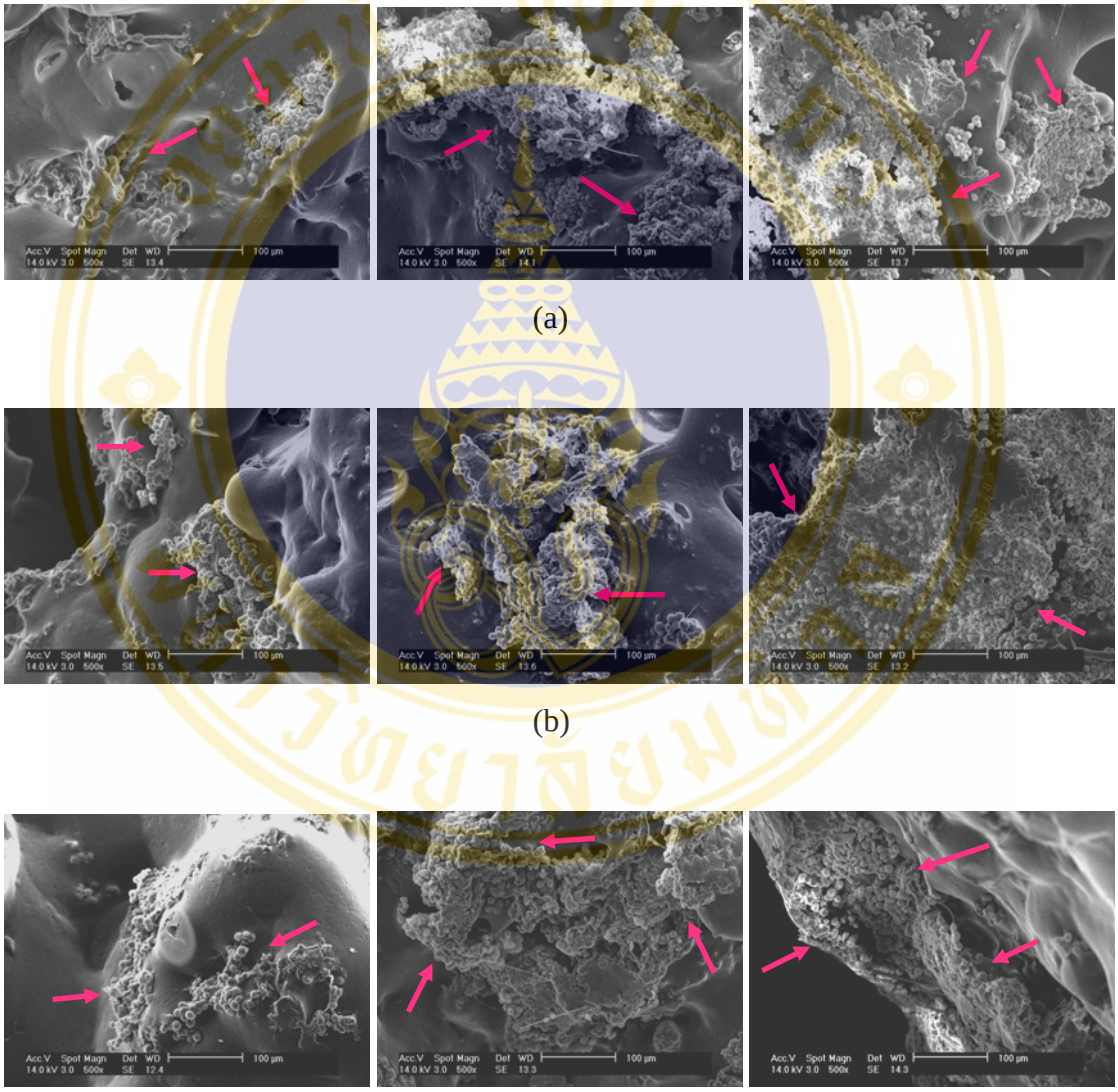


Figure 4.10 FTIR spectrum of chloroform.

4.3The possibility to use the scaffold for tissue engineering

The in vitro studies are conducted with Hepatocyte cells. The morphology of the cells on the different modified scaffold was also analyzed by SEM as shown in figure 4.11 (a)-(c).



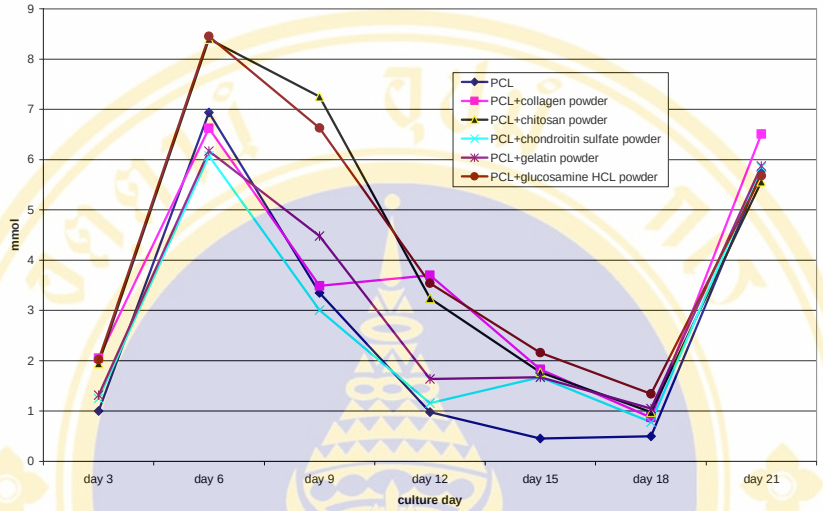
(a)

(b)

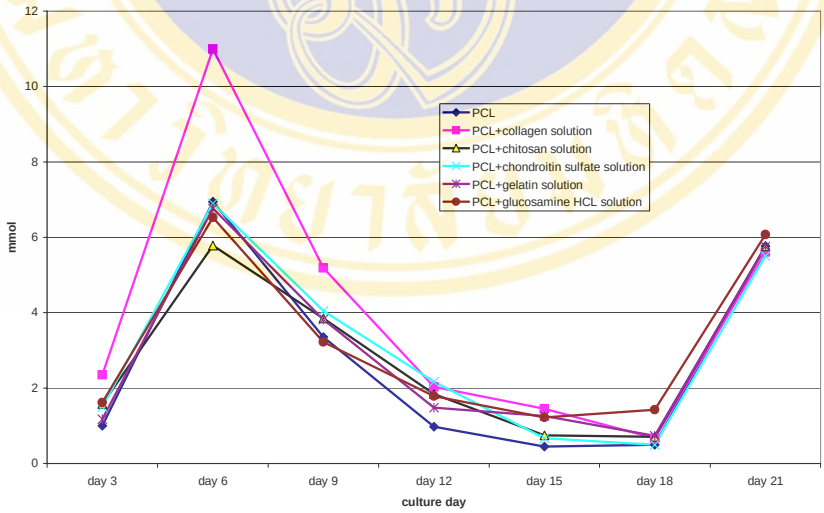
(c)

Figure 4.11 Cell morphology on the surface of PCL composite scaffold at 7, 14 and 21 days, respectively. (a) PCL scaffold, (b) PCL mixed with collagen solution, (c) PCL blended with glucosamine HCL powder.

The metabolic activity of the cells on various scaffold were investigated by measuring the secretion of lactic acid, at different time points. The results show in figure 4.12.



(a)



(b)

Figure 4.12 The quantity of lactic acid from Hepatocyte cells on scaffolds.
(a) scaffold blended with organic powder,
(b) scaffold mixed with organic solution.

CHAPTER V
DISCUSSION

5.1 PCL scaffold fabrication

The study of solvent effects on PCL scaffold preparation showed that chloroform can dissolve PCL at both low polymer concentration (5%) and high polymer concentration (10%) (see Table 4.1). By changing the solvent to be DCM, a slower dissolution of the polymers was obtained in both cases when comparing to that of chloroform. However, this dissolution rate was still faster than that of acetone, which took much longer period of time to dissolve 5% PCL polymer. This indicate that the PCL polymer tend not to dissolve in acetone especially when the concentration of polymer increased to 10%, PCL polymer can partially dissolved even for a longer period of time. Therefore, the chloroform shows the promising of being a good solvent for PCL using in this study followed by DCM and acetone respectively.

The effect of solution concentration on surface morphology of solvent casting film was shown in Figure 4.1. In this study, chloroform was used as a solvent. The 5% PCL solution cast sample showed the smooth surface with elongated aggregates and partially fused together, leaving traces of narrow voids on the surface. In contrast, the 10% PCL sample showed a rough surface with some visible pores. This can be explained by increasing the concentration of polymer, the mobility and orientation of the PCL molecule was limited which impeded the uniform spreading of the polymer molecule on the substrate before it was dried. A sample prepared at the concentration lower than 5% yields brittle film. However, at the concentration of higher than 10% the polymer solutions were very viscous, therefore, a film can not be prepared. The suitable concentration to fabricate PCL scaffold in this study was found to be between 5-10% w/v.

According to the effect of solvent used in the previous study the result showed that chloroform, DCM, and acetone are the prefer solvent for PCL respectively. Thus, the chloroform was chosen to be the solvent for scaffold fabrication using electrospinning technique. However, using chloroform as a solvent, a film sample could not be obtained possibly due to the low dielectric constant of solvent used. Therefore, the DCM had been used as the solvent instead. The results of fiber obtained by electrospinning are shown in figure 4.2 (a), (b), (e), (f). These results showed lots of beads containing in the sample especially in figure (a) and (b) where the optimal spinning conditions at 0.8 ml/h injection rate and 6.5 kV accelerating voltage. These samples shown clusters of polymer mixed with fibers causing by the drops of polymer solution on the collector, the fiber diameter are ranging from 0.311-5.316 μm. A similar result was also obtained at a higher injection rate at 1.1 ml/h with the accelerating voltage of 8.5 kV. The average fiber diameter of spun fibers was about 0.0069 μm with lots of beads containing in the sample (see figure 4.2 (e)-(f)). These results agree well with the study done by Zong XH [48] which showed that the increasing in accelerating potential, the injection rate must be increased as well as the amount of bead-like polymer will also increased. On the other hand, the fiber diameter will be decrease. By adding dimethyl formamide (DMF) into DCM solution, the spun fibers contained less beads (see figure 4.2 (c) and (d)). The optimal spinning conditions for DMF/DCM solvent was 1.0 ml/h injection rate with the accelerating voltage of 11 kV. More uniform fibers with an average diameter of 0.176 μm were formed. The number of beads found in the sample was consider to be smaller quantity. This phenomenon was agree well with Lee KH's [26] work which reported that by increasing DMF volume fraction in the polymer solution the surface tension and viscosity of the solution decreased, while conductivity and dielectric constant of the solution increased. Therefore, the electrospun fibers showed a more uniform distribution with less beads produced. Moreover, by varying the distance between the needle tip and the collector, the fiber diameter was decreased with increasing the distance. In addition to the prescribe parameters; solution viscosity, conductivity, surface tension, injection rate, electrical potential, distance between the needle tip and the collector, Doshi J [15] also reported that the hydrostatic pressure in the capillary,

ambient parameters including temperature, humidity, and air velocity in the electrospinning chamber also influence to the quality of electrospinning fibers.

Although this technique can produce fibers in nanometer scale which can increase the surface area of the scaffold greatly. The resulted products have very fine pores, which could prevent water absorption and inhibit cell penetration into the scaffold. These large numbers of involving parameters together with the time consuming process, that make the electrospinning scaffold become less attractive to produce comparing to the other scaffold preparation method. Moreover, forming a complex design pore structures is also much more difficult than in other scaffold fabrication processes. Consequently, this technique may not suitable for this study. Therefore, the solvent casting/salt leaching method was then selected to use as an alternative method to fabricate the scaffold in this study.

The results of scaffold prepared using solvent casting/salt leaching technique with varying salt concentration showed that the porosity of the scaffold can be tuned by modifying the salt concentration. In this case, two weight ratio of PCL:NaCl were used; 1:10 and 1:25. The SEM micrographs of prepared samples were shown in Figure 4.3. The sample prepared at low amount of porogen showed a rough dense surface with low porosity as seen in Figure 4.3 (a). By increasing NaCl ratio from 1:10 to 1:20, the porosity of the scaffold was increased which agree well with the experiments done by Reignier J [35]. The sample prepared at 1:25 PCL:NaCl revealed the visible pores (Figure 4.3 (b)). The size of these pores varied considerably throughout the scaffold from tens to hundreds micrometers and depended on the size distribution of the salt particles used. The morphology of the sample demonstrated good interconnection with the porous matrix, indicating that no skin layer was formed on the surface of the salt particle. The absence of salt crystals suggested that the dissolution of the salt crystals was completed.

It clearly appeared that the prepared scaffolds possess a very good interconnectivity between the pores, and that strongly desired in tissue engineering. It may be desirable to indicate the size of the pore opening and porosity of the samples in order to evaluate the quality of the scaffolds. However, the porosity of the scaffolds

was not determined directly by pore size and porosity measurement but the water absorbability test could indicate the porosity of the samples without losing any significance.

5.2 The PCL scaffold properties

As mentioned previously, cell adhesion onto a scaffold depends on various parameters such as; pore structure, total surface area, and surface chemistry of scaffold. This surface chemistry may play an important role on cell attachment during cell culturing process especially on the synthetic scaffold, which usually possesses strong hydrophobicity property. Therefore, the modification of scaffold surface properties by blending with various organic substances to the polymer solutions was carried out and the morphology of results composite films were shown in Figure 4.4. The surface of PCL film was rough with some visible pores (Figure 4.4 (a)). Adding glucosamine HCL, chondroitin sulfate, or gelatin powders, the composite films showed smoother surfaces than that of the pure PCL film. However, the surface morphology of these samples showed the traces of grain boundary on the surface throughout the sample especially in the cases of glucosamine HCL and gelatin added (Figure 4.4 (b), (e), and (f)). By blending chitosan with PCL, the casted film was rough and occupied by chitosan flakes over the film surface (Figure 4.4 (c)). The PCL-collagen composite film exhibited the roughest surface with particle trapped inside the film surface, some visible pores and cracks were found on the sample (Figure 4.4 (d)). This study showed that the surface morphology of the composite films were varied depending on the organic additives added.

The results study from the effect of organic additives on the contact angle of PCL composite films were summarized in Table 4.3. The results showed that the pure PCL film had the highest advancing contact angle of 99.39° $\pm$ 1.84 (n=10) comparing to the composite film. All composite films showed significant lower advancing contact angles ($p<0.05$). The contact angle measured on PCL blended with chondroitin sulfate, gelatin, glucosamine HCl had contact angles at 59.50° $\pm$ 5.72 , 61.73° $\pm$ 7.51 and 56.80° $\pm$ 9.96, respectively. However, the value of contact angle for collagen-PCL composite film was 20.38° $\pm$ 2.32 which reduced greatly compared to other composite films ($p<<0.05$). This could indicate the effect of the organic presence on the

hydrophobicity of the sample. Although, the water contact angle can explain the hydrophobicity of a surface, it is not solely varied with the organic substance added. The water contact angle also relates to surface roughness. According to Wenzel [47], the measured water contact angle is inverse proportional to the surface roughness as shown in equation (2). Therefore, the examination of surface roughness on each sample was performed using atomic force microscopy (AFM). The results in Table 4.4 showed that the pure PCL film had the roughness (Ra) value of 79.035 $\pm$ 0.68 nm (n=5). By adding organic powders, the surface roughness increased drastically ($p<<0.05$). The PCL composite films blend with chondroitin sulphate, gelatin, glucosamine HCl and collagen had the Ra value of 163.99 $\pm$ 0.88 nm, 278.84 $\pm$ 1.51 nm, 325.13 $\pm$ 0.78 nm and 386.82 $\pm$ 0.73 nm, respectively. These results of roughness measurements indicated that the water contact angles measured from the previous study may not be explained the hydrophobicity of the surface due to the influence of the roughness. Since the surfaces of the films were not smooth, the interface between water droplets and the polymer film were not symmetry especially in collagen-PCL composite sample, which had the highest roughness. This may be the reason why the composite collagen-PCL yielded the lowest water contact angle.

In order to accommodate the roughness effect to the water contact angle measurement, the organic additives were dissolved and mixed with PCL solution. The PCL composite solutions were cast as films, performed water contact angle, and surface roughness measurement. By changing organic additives from solid powders to solutions the surface roughness of film samples were reduced to about the same as pure PCL film (79.035 – 87.074 nm). This resulted in reducing the influence of surface roughness on water contact angle measurement. Therefore, the water contact angle measured from the new composite films should depict the effect of various types of organic additives to hydrophobicity of the film. The results showed that when organic solutions were used instead of organic powders, all PCL composite films with organic solutions had increased in water contact angle values compared to samples prepared by organic powders. The contact angles of these samples were very closed to that of pure PCL film (92.00° – 98.91°) with the ($p>0.05$) exceptional for the PCL-collagen solution composite film, which showed the significantly lowest ($p<0.05$)

contact angle of 70.55°. These results had confirmed that the surface roughness of the sample can manipulate the contact angle measurement. However, the variations of contact angle on a surface depend not only on the morphological modification of the modified surface, but also on the chemical modifications of the modified surface. In this study, most of PCL composite films mixed with organic solutions had no significant difference roughness and no significant decreasing the measured contact angle except collagen-PCL composite. Therefore, it may be assumed at this moment that collagen can enhance the surface wettability of the sample.

In order to used PCL composite for tissue reconstruction, the porous scaffold must be prepared. The different scaffolds with various organic additives (both solid powders or solvents forms) were prepared using solvent casting/salt leaching technique. The morphology of each scaffold was examined and the results were shown in Figure 4.5 and 4.6 for the scaffold blended with organic powder and mixed with organic solution respectively. The PCL scaffold revealed the interconnected pores (Figure 4.5 (a)) with pore size between 58.711-175.469 μm. Adding organic powders, the composite scaffold displayed the reduction in pore size (Figure 4.5 (b-f)). The average size of these pores varied considerably throughout these scaffolds from 49.274-110.681 μm. The morphology of the polymer wall on composite PCL mixed with organic powders had sheet-like structure containing micropores which were dispersed over the wall surface. Figure 4.6 showed the SEM images of the composite scaffolds prepared from PCL solution mixed with organic solution. These scaffolds exhibited similar structure than that of the scaffold blended with organic powders with the average pore size of 102.040-183.879 μm (see Figure 4.6 (b-f)). However, the overall morphology of the samples prepared using organic solutions had looser structure compared to that of PCL/organic powders composite. This may due to the presence of water during casting process, which in turn evaporated and leaving large pores on the polymer matrix. The structure of PCL-glucosamine HCL solution and PCL-chondroitin sulfate solution composite scaffolds pores were connected to each other by the thin (~10 μm.), smooth strands walls, while the PCL-chitosan solution, PCL-collagen solution, and PCL-gelatin solution composite scaffolds were connected by solid polymer walls with the micropores on the polymer matrix. This possibly due

to the influence of the organic powders and organic solutions on the solidification process by affecting the evaporation rate, phase separation rate, and crystallization rate of PCL solution. However, there is no clear explanation of these effects on the scaffold formation and they should be investigated in the future.

To evaluate the porosity of the scaffolds, water absorption ability on each sample were conducted. This measurement is important to any biomaterial because it not only depicted the porosity of the scaffold but also explain the ability of cells to be attached or absorbed into the scaffold. The % water uptake of the composite scaffolds prepared using the organic additives in both powders and solution forms were measured (based on the method of Park SN [32]). The results summarized in table 4.6 showed that the water contact angle decreased by adding organic additives especially when the organic solutions were used. The results showed that the composite scaffold mixed with organic solution had higher % water uptake than those of the composite scaffold blended with organic powder. The unmodified PCL scaffold had % water uptake of 86.04 while the composite scaffolds blended with chitosan, gelatin, and collagen powder showed a slightly increasing in % water uptake to 88.17, 88.87 and 89.86 respectively. However, the opposite results were obtained in the PCL scaffold blended with glucosamine HCl and chondroitin sulfate powders (79.53, 81.20). The addition of organic solutions showed a larger increasing in % water uptake in all samples, possibly due to the higher porosity of the scaffold which were seen from the micrographs that theses scaffolds contains small pores on the polymer wall. It is interesting to note that the PCL scaffold mixed with both collagen powder and collagen solution gave the highest % water uptake among each system. Moreover, all sample prepared by mixing organic solutions with PCL showed higher % water uptakes compared to the scaffold with dry organic powders. These results may due to a higher hydrophobicity or higher porosity of the composite scaffolds, which have to be delineated by, carried out a study on the controlled porosity composite scaffolds. Although, the % water uptake of the most composite scaffolds showed a higher value than that of the pure PCL scaffold, they gave no statistical significant different value ($p>0.05$) of %water uptake of the composite scaffolds compare to that of pure PCL scaffold.

Finally, the enzymatic degradation test followed Gan ZH's [18] condition, was performed on all prepared scaffolds. Degradation in both two groups of scaffolds was characterized by %weight loss after immersed to lipase solution. Figure 4.7 showed the weight changes of the scaffolds blended with organic powders and organic solutions respectively. The results demonstrated a linear relationship between the % weight loss and time, which agreed with the random hydrolytic chain scission of the ester linkages mechanism proposed by Sun H [40]. It can be seen in this study that the degradation was nearly complete within the short period of time (15 days) compare to a reference that required 3 years to completely removal from the host body [33]. This could cause from the high concentration of the lipase solution used which has a direct effect on accelerating the degradation rate reported by Kim UJ [23].

The % weight loss of the composite scaffolds blended with organic powder was shown in Figure 4.7(a). All of the composite samples showed fast weight loss up to the day 9 then slightly decreased in weight loss afterward. The amount of the weight loss of composite scaffold was higher than that of PCL scaffold through the entire periods. All samples were nearly complete degraded within 15 days. However, there was no significant difference in % weight loss of the entire samples compare to the pure PCL scaffold. The % weight loss of the composite scaffold mixed with organic solution was shown in Figure 4.7(b). The composite scaffolds and the PCL scaffold showed a similar weight loss behavior through the test periods. There is no obvious difference in the weight loss among the entire samples. After 15 day of degradation test, the weight loss of the PCL composite scaffold in both two groups were not different from that of the PCL scaffold ($p>0.05$). Indicating that adding organic additive to the polymer solution before preparing the composite scaffold does not alter the degradation mechanism of the polymer and no influence on the degradation rate of the PCL sample as well. Although, it was seen that a higher % weight loss can be obtained by blended PCL with some organic powders, the differences were not significant and it will require more intensive study on biodegradation mechanism in the future. Angele P [4] suggested that the other factors that may affect the enzymatic degradation susceptibility of the scaffold are the swelling ratio and the surface area. The differences in the primary structure of scaffold also resulted in significant a

different physicochemical property because of the enzymatic activity mainly affects the matrix surface.

According to the solvent casting/salt leaching method which required the use of a proper solvent to dissolve the polymer, the presence of organic solvent resided in the porous scaffold may be harmful to adherent cells or nearby tissues. To ensure that there is no residual solvent presenting in the polymer, an FTIR study on prepared sample was performed.

Figure 4.8 showed the absorption spectrum of PCL melt casting film in the range of 600-2000 cm^{-1} region. The characteristic bands around 1728-1734 cm^{-1} for the carbonyl stretching were assigned to PCL polymer. The characteristic peaks at 1171 cm^{-1}, 1239 cm^{-1} and 1293 cm^{-1} were assigned to symmetric C-O-C stretching, asymmetric C-O-C stretching and C-O, C-C stretching in the crystalline phase respectively. The strong absorption bands of the spectrum and their assignments based on Elzein T (2004) were summarized in Table 2.3. Together with the FTIR spectrum of pure chloroform showed the strong absorption at 761.7 cm^{-1} corresponding to the C-Cl stretching mode (see Figure 4.10), the appropriate PCL samples to be use as the biomaterial should not have this absorption band in their structure. The FTIR results on prepared film after 1 and 3 days of evaporation showed that the films had similar absorption patterns to that of pure PCL film. They also contain no residual chloroform in the PCL scaffold even for the sample with the shortest evaporation time.

5.3 The evaluation of scaffold for tissue engineering

The cell morphology of hepatocyte cells on the scaffolds was examined after 7, 14 and 21 days of culturing. The SEM revealed that hepatocyte cells attached and proliferated on the scaffolds. More exciting finding was that the hepatocytes not only adhered on the scaffold surface but also grew into the pore. The cells were observed to be extended and spread over the surface. Regardless of the different surface chemistry, no differences of morphology were found between cells cultured on the composite PCL scaffolds blended with organic powder and composite PCL scaffolds mixed with organic solution. Figure 4.11 (a)–(c) showed that at the day 7 of cultivation, the cells grew and expand in form of aggregates, where all cell are still in round shape.

Although, the proliferation was not obvious, the cells are regularly grown in and onto the scaffolds. As the experiment proceed to 14 days, the cells are increasing in their numbers significantly and almost reached approximately 50% confluence on the scaffold compared to that of the day 7. The SEM revealed that the cells were retained their spherical shape and tended to form clusters without spreading. An interesting observation was that at the end of day 21, the scaffold surface was observed to be nearly complete covered with a continuous hepatocyte monolayer and showed approximately 70% confluence. Krasteva N [24] was explained that the wettability of substrate has an influence on hepatocyte morphology, viability and function, such a relationship was not investigated in this study. Since the fate of the cells on the biomaterial depended on its attachment (its initial contact) to the surface, once attached it proliferated. These results indicated a well attachment and proliferation of the hepatocytes within the scaffold. On the other hand, Krasteva N [24] suggested that when hepatocytes had a weak contact with the substrate they may survive by anchoring to each other, forming tight cell–cell contacts, which may also provide better conditions for some cellular functions, such as protein synthesis. It was interesting to find that culturing cells on different surfaces did not cause any change in their nature and their usual formation. However, it was difficult to determine the exact number of cells on the surfaces of seeded scaffold, due to the surface texture of the material.

To evaluate the influence of the organic additives in the scaffolds that may affect the cell growth, the metabolic activity of hepatocyte cells on the various scaffolds was investigated over 21 days period. Lactic acid discreted by the cells was assessed in culture medium at controlled environment. The collection of the sample medium is performed in every 3 days. Values were calculated by subtracting the amount of lactic acid measured in cell culture medium. The results were shown in Figure 4.12 (a) and (b) for the composite scaffolds with organic powder and composite scaffolds with organic solution respectively. All samples revealed the similar trend of lactic acid discrete over the test period. The increasing quantity of lactic acid during day 3 to day 6 was obtained on all the scaffolds. The increasing of lactic acid to 1.00-2.35 mmol at day 3 and 6.07-8.45 mmol at the end of day 6 indicated that proliferation

of hepatocyte cells on the scaffolds were occur. Although, the PCL composite scaffold mixed with collagen solution exhibited the highest quantity of the lactic discrete (10.99 mmol), the statistical difference to that of other samples is insignificant ($p>0.05$). The quantity of lactic acid collected at day 6 to day 18 showed a significant decreasing of lactic acid by approximately 90% from that of day 3 to 0.5-1.43 mmol ($p<0.05$). After 18 days of culture, the amount of lactic acid in all specimens increased to 5.51-6.51 mmol indicating that the number of the cells were increased, which could be contributed by cell differentiation. Nevertheless, further researches on the interaction between the hepatocyte and the composite scaffold should be studied to evaluate the scaffold properties for the practical using.

CHAPTER VI
CONCLUSION

The preparation a scaffold for utilizing in tissue engineering involves with various factors. This study showed that chloroform is a proper solvent for PCL scaffold fabrication via solvent casting and evaporation method. The suitable polymer concentration to fabricate PCL scaffold is 10% w/v. The alternative electrospinning technique to fabricate PCL scaffolds can be performed using dichloromethane (DCM) mixed with dimethly formamide (DMF) in steady of chloroform. The proper polymer concentration in mixed solvent is 8 %w/v. The mixed solution helps to form fiber due to its higher dielectric constant which can create the fiber in the range of 100-200 nm. The fiber diameter can be decreased with increasing injection rate or accelerating voltage. The proper condition to perform the electrospinning process is the injection rate of 1.0 ml/h, 11 kV accelerating voltage with the distance between the needle tip and ground collector of 15 cm. However, the resulted scaffolds prepared by electrospinning have limiting in water absorption capability possibly due to the surface tension of the spun fibers and the pore diameter of the scaffolds. Therefore, the solvent casting/salt leaching technique is employed for scaffold fabrication due to its simplicity in processing and porosity controlling. The 10 %w/v PCL solution concentration was used with NaCl particles and the porogen. The results showed that the porosity increased as increasing NaCl amount. In this study, varieties of composite scaffolds have been prepared. It is found that blending organic additives into the PCL solution in solvent casting process can decrease the water contact angle of the prepared scaffolds. In this study, the PCL/collagen composite film gave the lowest advancing water contact angle of 20.38° compared to the other composite scaffolds while the pure PCL film showed relatively high advancing water contact angle of 99.39°. However, the addition of the organic powders may not affect the hydrophobicity of the surfaces but it also introduced some roughness on the samples,

which could also lower water contact angle as shown by the highest surface roughness of 386.82 nm on PCL/collagen film. This also confirmed by the surface roughness and water contact angle measurements on PCL/organic solution composite films. The PCL/collagen solution sample still gives the lowest water contact angle, while the other composite films mixed with organic solutions have water contact angles in the range of pure PCL film while the surface roughness of the prepared films was reduced to approximately the same as that of the pure PCL film. Therefore, the water contact angle of PCL/organic solutions could indicate true water contact angle effect solely on the surfaces without the influence of surface roughness. The SEM micrographs of the composite scaffold show that adding organic substance to the PCL solution does not change the macrostructure of the scaffold. The % water uptake of the scaffold blended with organic powder is about 81.00-90.00 % which is lower that that of the scaffold mixed with organic solution (90.00-94.00 %) implying that the composite scaffold prepared using organic solution yield a higher porosity than that of PCL/organic powders. The enzymatic degradation rate on PCL/organic powders scaffolds using lipase solution show a slightly faster degradation rate than that of the scaffold mixed with the organic solution and the entire samples are degraded nearly complete within 15 days. The evaluation of prepared scaffold for tissue engineering includes cell morphology and cell metabolic activity on different composite scaffolds. The results showed no obvious relations between the wettability of the scaffolds and their ability to support cell adhesion and function. However, hepatocyte cells can attach on all substrates and tend to make aggregates in vitro. The SEM results show that the cell growth on scaffolds are sphere aggregates and spread over the surface of the scaffolds. The cell morphology and cell metabolic activity exhibited no significant different results between the entire specimens.

Although, the addition of organic additives in PCL polymer does not give impressive results in terms of cell culture and proliferation, some of the organic substances added into the PCL polymer such as collagen may change hydrophobicity of the polymer by lowering the water contact angle compare to the other substances. However, the change in water contact angle may not adequate to evaluate the quality

of a prepared scaffold. Therefore, the effects of other parameters such as porosity, surface area, and pore size of the scaffolds must be evaluated in the future.

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APPENDIX

APPENDIX A

Measurement Data on PCL films and Scaffolds

List of sample ID

1. PCL film
2. PCL:chitosan flake film
3. PCL:chondroitin sulphate powder film
4. PCL:gelatine powder film
5. PCL:glucosamine HCl powder film
6. PCL:collagen powder film
7. PCL:chitosan solution film
8. PCL:chondroitin sulphate solution film
9. PCL:gelatine solution film
10. PCL:glucosamine HCl solution film
11. PCL:collagen solution film
12. PCL scaffold
13. PCL:chitosan flake scaffold
14. PCL:chondroitin sulphate powder scaffold
15. PCL:gelatine powder scaffold
16. PCL:glucosamine HCl powder scaffold
17. PCL:collagen powder scaffold
18. PCL:chitosan solution scaffold
19. PCL:chondroitin sulphate solution scaffold
20. PCL:gelatine solution scaffold
21. PCL:glucosamine HCl solution scaffold
22. PCL:collagen solution scaffold

Table I Contact angle

Sample ID	Contact angle (°)										
	1	2	3	4	5	6	7	8	9	10	SD
1	99	99	98	102	97	97	102	101	99	100	1.84E+00
2	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	-
3	62.5	57	64.5	49	59	53	64	56	63	67	5.72E+00
4	69	67	67	67	65	48	63	65	55	51	7.51E+00
5	58	43	55	59	70	70	63	48	60	42	9.96E+00
6	20	25	23	20	20	20	16	20	20	20	2.32E+00
7	94	95	99	98	95	99	97	98	93	99	2.26E+00
8	93	100	95	98	102	100	84	75	74	99	10.54E+00
9	84	100	90	111	100	100	104	108	96	96	8.01E+00
10	95	97	96	98	98	97	96	97	98	95	1.16E+00
11	60	69	78	73.5	73	80	68	67	67	70	5.82E+00

Table II % Water uptake

Sample ID	*Wd*	*Ws*	*Ws- Wd*	$\frac{(Ws-Wd)}{Ws}$
12	0.0187	0.1340	0.1153	0.8604
13	0.0333	0.2816	0.2483	0.8818
14	0.0154	0.0814	0.0661	0.8113
15	0.0209	0.1856	0.1647	0.8874
16	0.0199	0.0972	0.0773	0.7953
17	0.0198	0.1953	0.1755	0.8985
18	0.0132	0.1774	0.1641	0.9253
19	0.0160	0.1681	0.1521	0.9048
20	0.0142	0.1882	0.1740	0.9247
21	0.0161	0.1651	0.1490	0.9027
22	0.0139	0.2228	0.2089	0.9375

Table III % Weight loss

Sample ID	Weight loss (%), day					
	1	3	6	9	12	15
1	5.87	14.58	34.97	48.20	70.81	89.54
2	34.48	56.08	79.13	93.65	96.78	98.13
3	29.33	61.40	70.90	83.50	99.40	99.76
4	28.60	50.21	53.44	57.37	85.76	92.67
5	32.43	56.09	69.56	76.56	85.12	93.07
6	34.03	54.29	70.44	94.25	98.69	99.01
7	11.24	26.34	37.26	52.12	89.38	92.48
8	12.04	16.26	38.76	57.30	82.39	96.06
9	18.87	40.02	47.71	73.78	86.49	91.55
10	19.12	38.03	40.55	50.79	91.34	90.06
11	8.85	33.47	43.21	60.17	73.38	90.22

Table IV Lactic acid quantity

Sample ID	Lactic acid quantity (mmol), day						
	3	6	9	12	15	18	21
1	1.00	6.94	3.35	0.98	0.45	0.50	5.77
2	1.95	8.40	7.25	3.24	1.77	0.98	5.56
3	1.25	6.07	3.01	1.16	1.67	0.78	5.85
4	1.32	6.17	4.48	1.64	1.67	1.05	5.87
5	2.02	8.45	6.63	3.54	2.16	1.34	5.68
6	2.05	6.62	3.49	3.70	1.83	0.87	6.51
7	1.58	5.78	3.85	1.84	0.75	0.71	5.76
8	1.50	6.90	4.05	2.17	0.67	0.50	5.51
9	1.18	6.77	3.82	1.48	1.26	0.73	5.75
10	1.62	6.52	3.22	1.79	1.22	1.43	6.07
11	2.35	10.99	5.19	2.03	1.45	0.67	5.61

APPENDIX B

Morphology of PCL samples

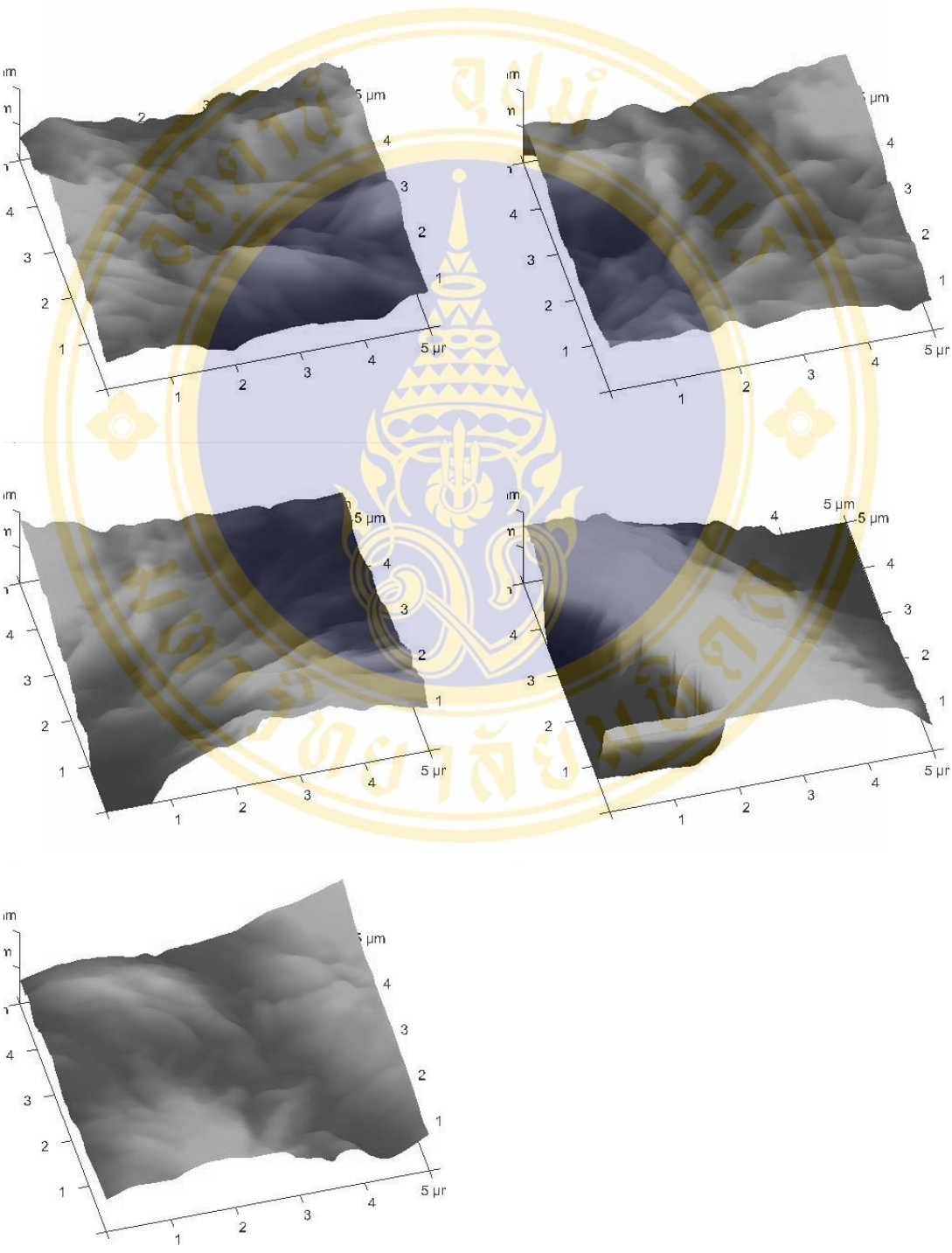


Figure B(1) AFM images of pure PCL film

Figure B(2) AFM images of PCL mixed with chitosan solution film

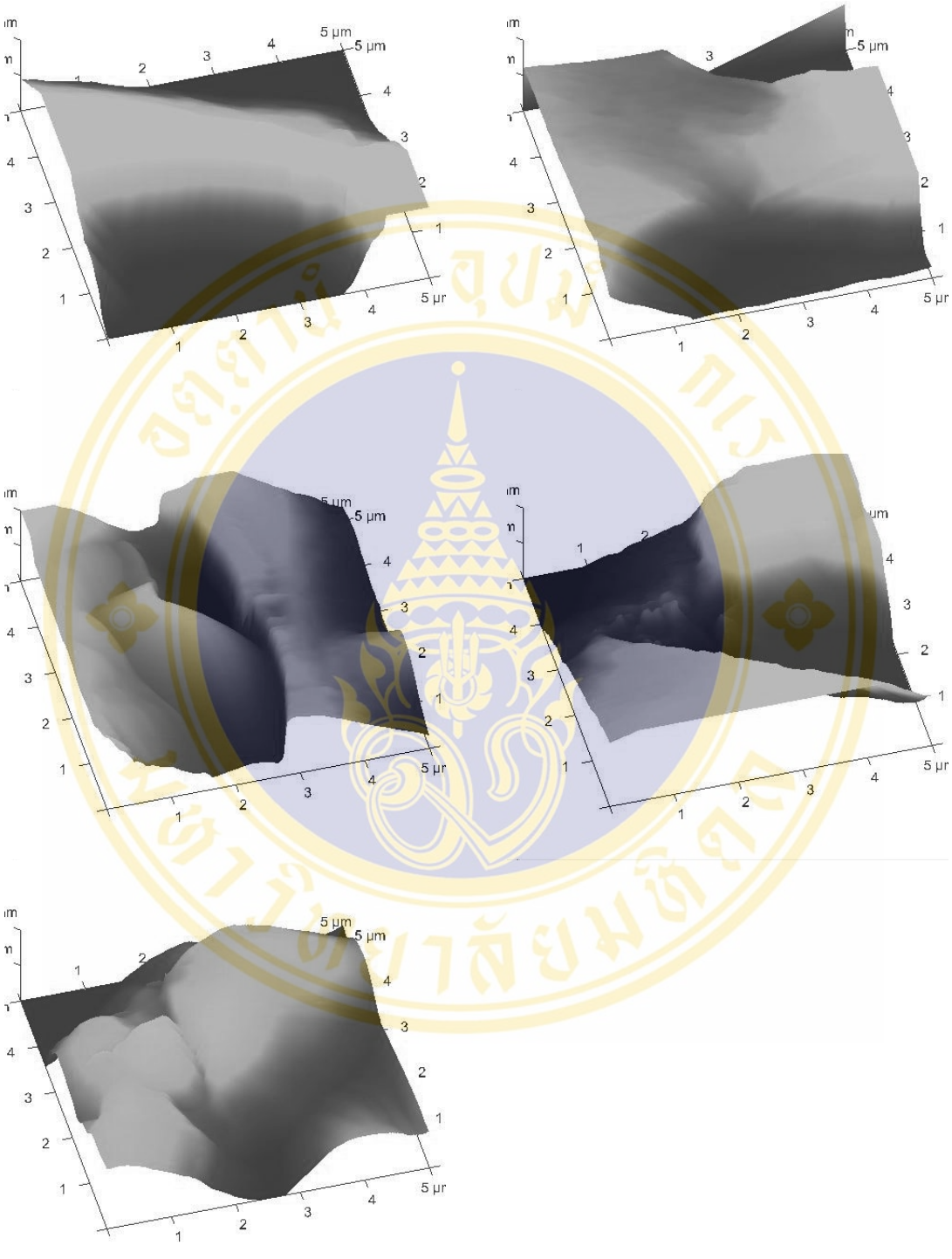


Figure B(3) AFM images of PCL mixed with chondroitin sulfate solution film

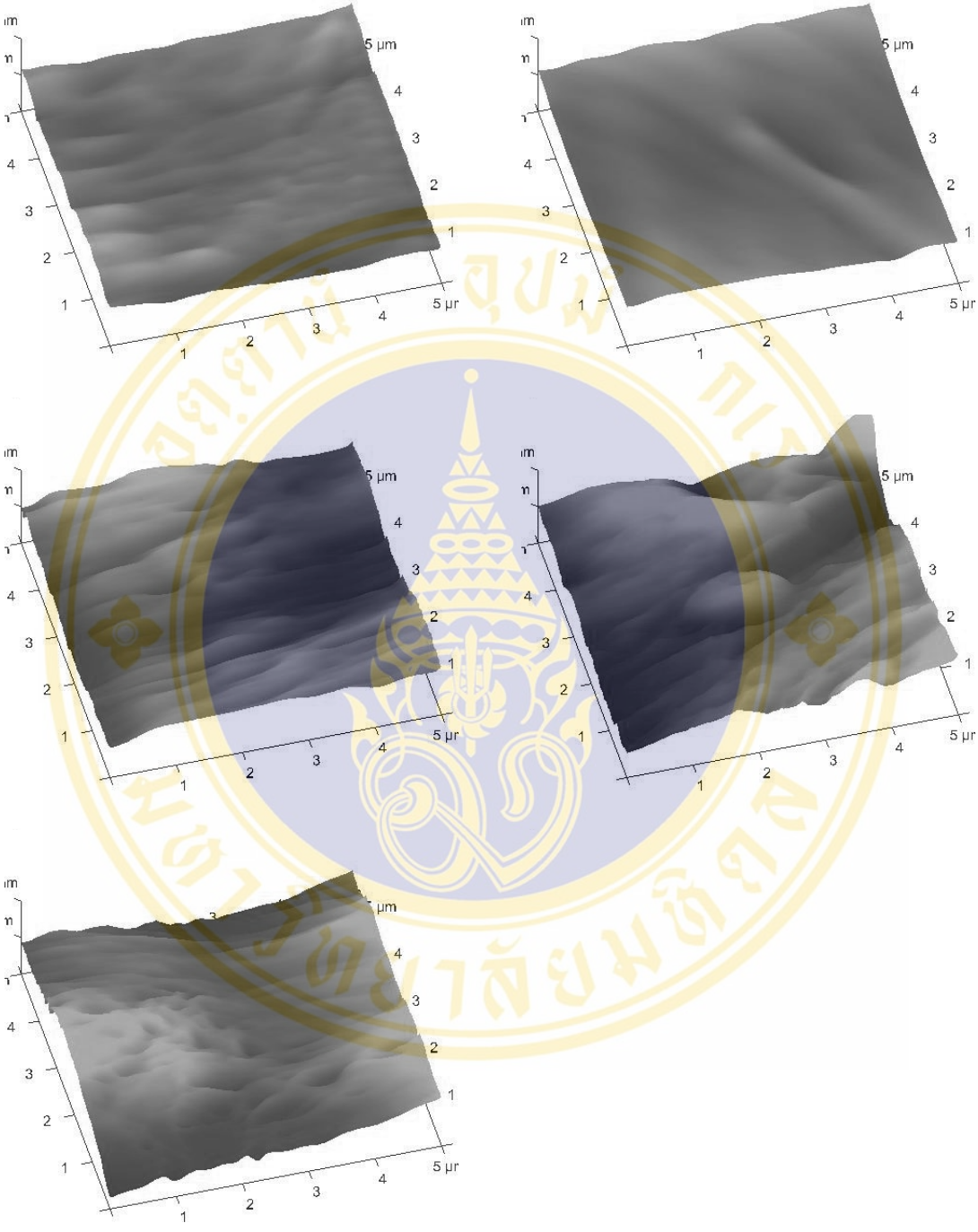
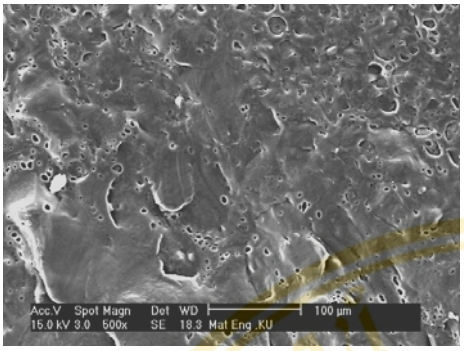


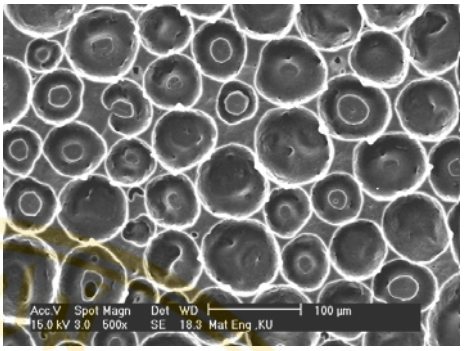
Figure B(4) AFM images of PCL mixed with collagen solution film

Figure B(5) AFM images of PCL mixed with gelatin solution film

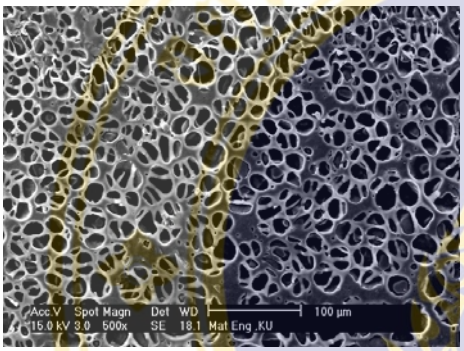
Figure B(6) AFM images of PCL mixed with glucosamine HCL solution film



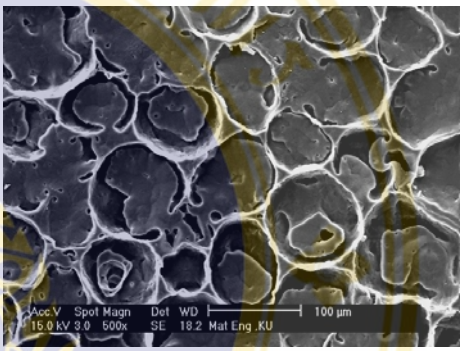
(a) PCL film



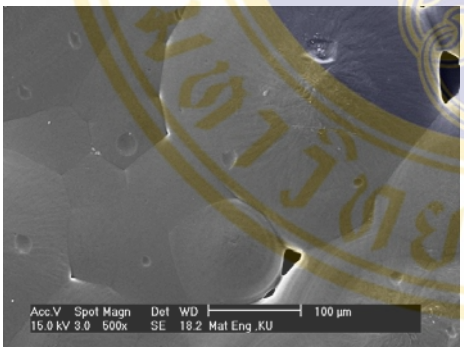
(b) PCL:Gelatin solution film



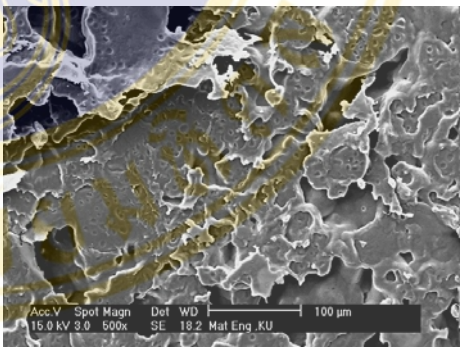
(c) PCL:Chondroitin sulfate solution film



(d) PCL:Chitosan solution film

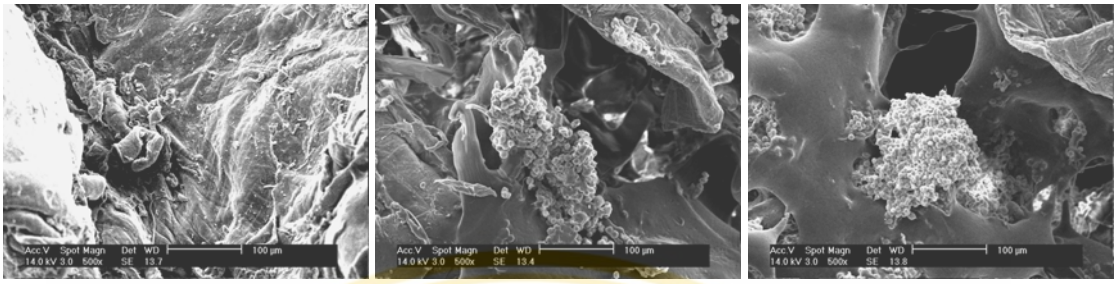


(e) PCL:Collagen solution film

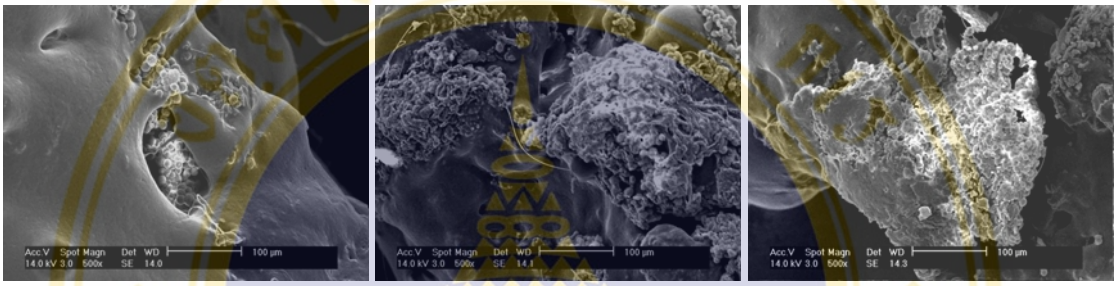


(f) PCL:Glucosamine HCL solution

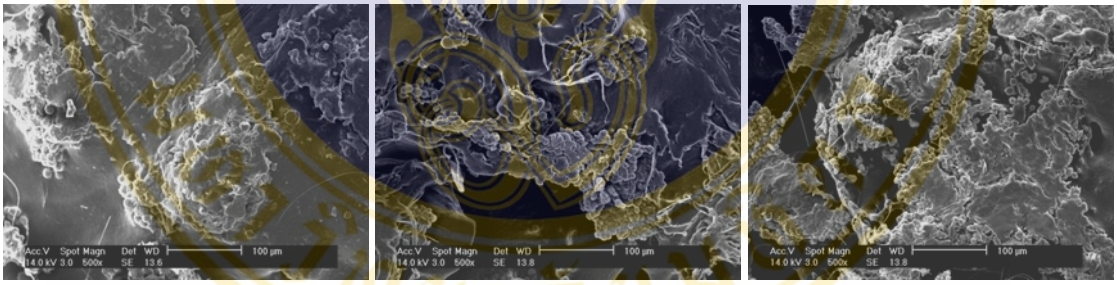
Figure B(7) SEM micrograph of PCL composite solution film.



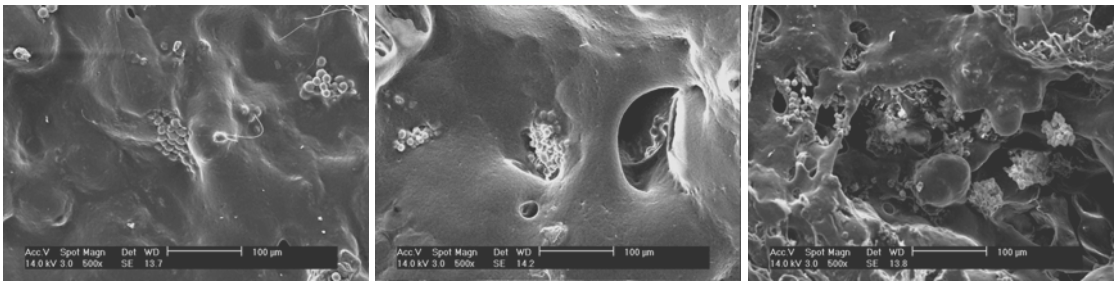
(a) PCL:Chitosan flake scaffold



(b) PCL:Chondroitin sulfate powder scaffold

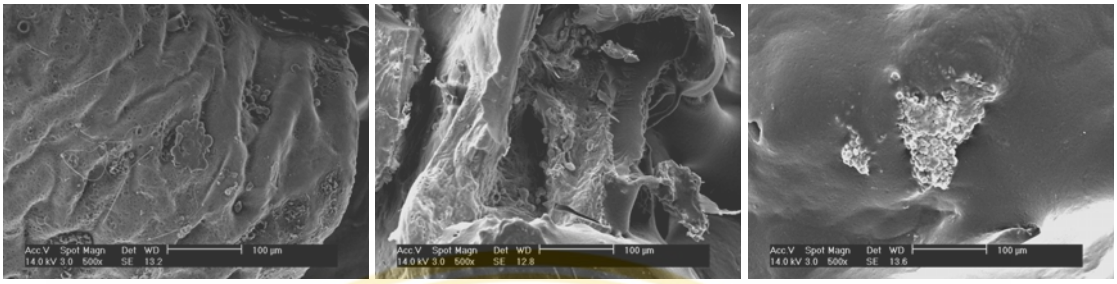


(c) PCL:Collagen powder scaffold

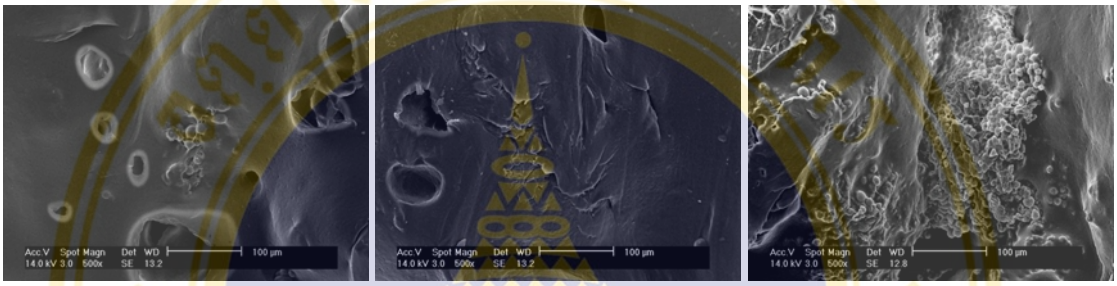


(d) PCL:Gelatin powder scaffold

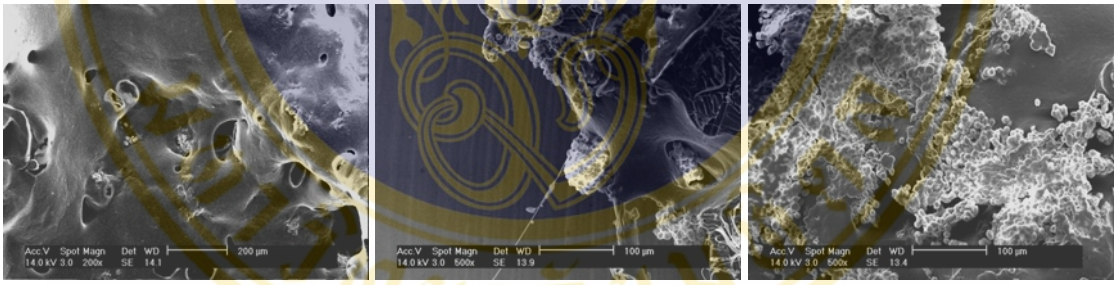
Figure B(8) Cell morphology on the surface of PCL composite scaffold at 7, 14 and 21 days, respectively.



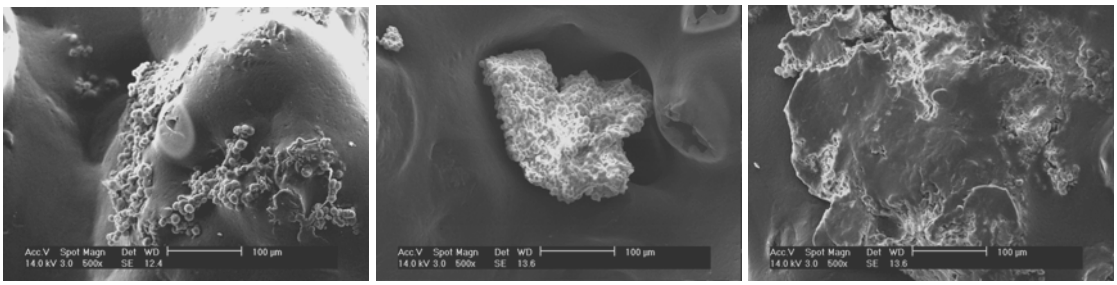
(e) PCL:Chitosan solution scaffold



(f) PCL:Chondroitin sulfate solution scaffold

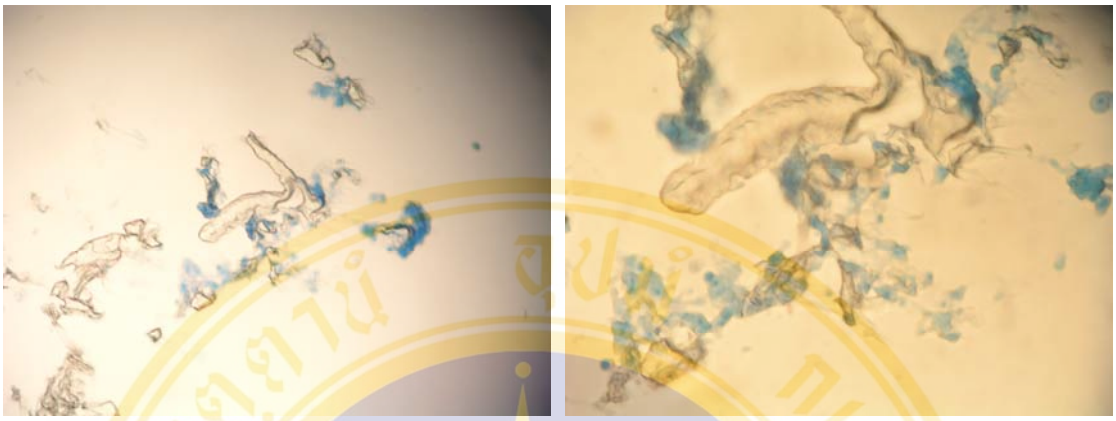


(g) PCL:Gelatin solution scaffold

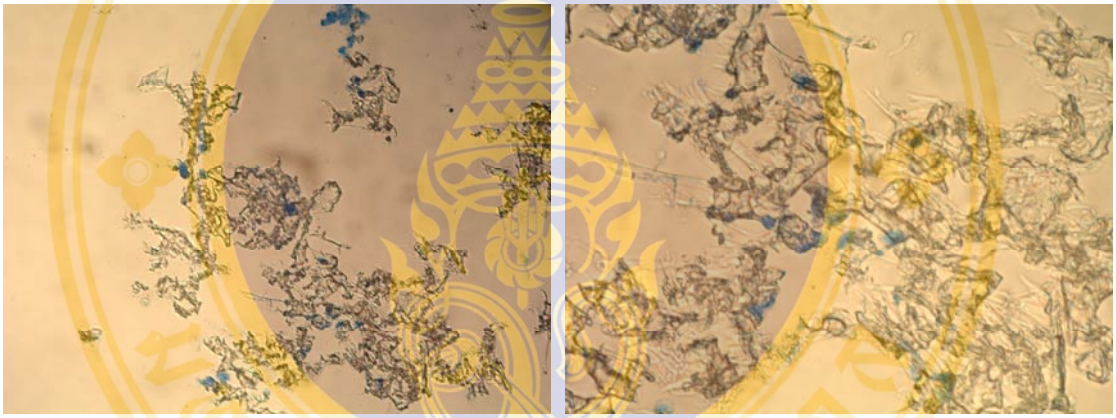


(h) PCL:Glucosamine HCL solution scaffold

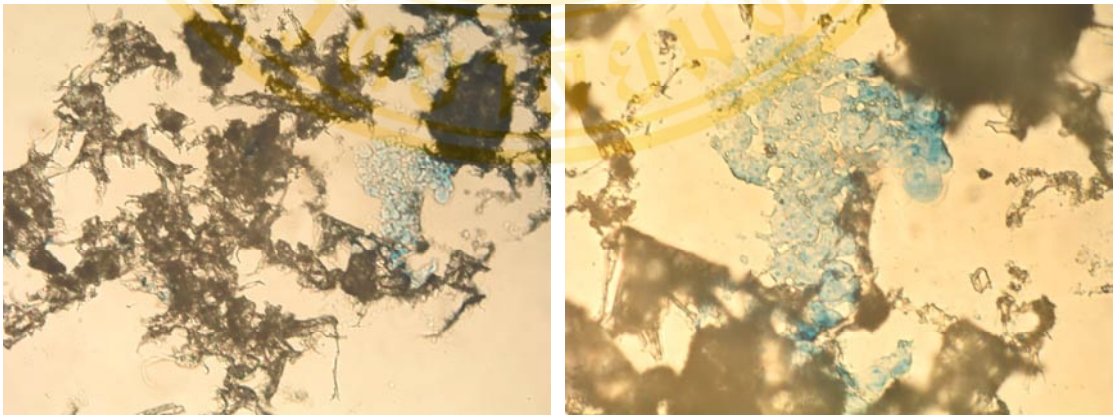
Figure B(8) Cell morphology on the surface of PCL composite scaffold at 7, 14 and 21 days, respectively. (continued)



(a) PCL scaffold

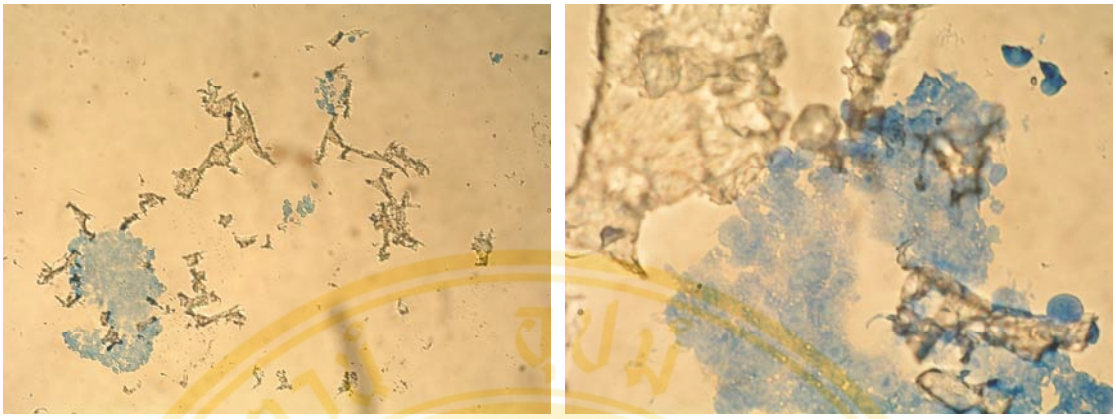


(b) PCL:Chitosan solution scaffold

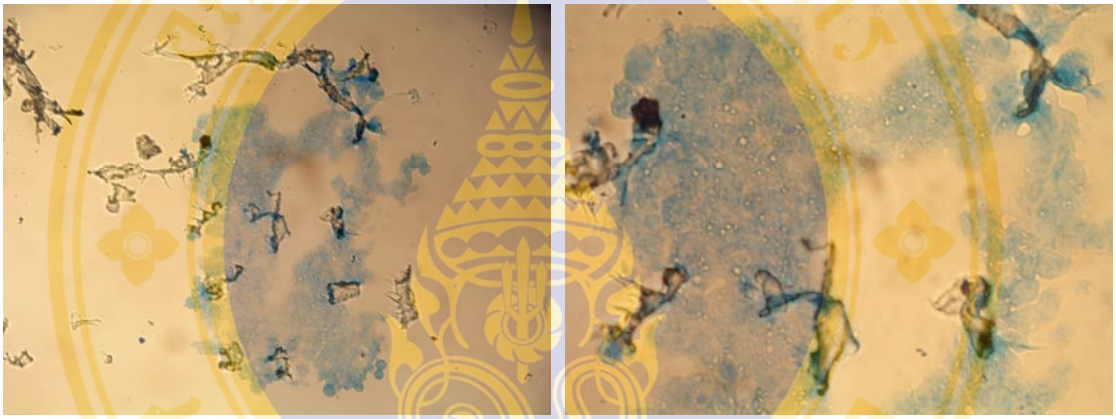


(c) PCL:Chondroitin sulfate solution scaffold

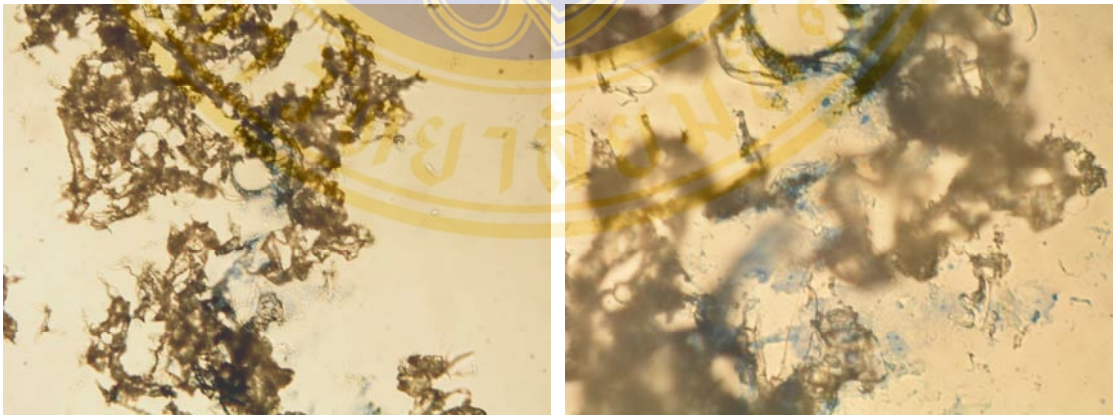
Figure B(9) Hepatocyte on PCL composite scaffold from Optical Microscope (magnification 20X, 50X)



(d) PCL:Collagen solution scaffold



(e) PCL:Gelatin solution scaffold



(f) PCL:Glucosamine HCL solution scaffold

Figure B(9) Hepatocyte on PCL composite scaffold from Optical Microscope (magnification 20X, 50X) (continued)

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