A STUDY OF ELECTRICAL CELL REPELLENT FROM CONDUCTIVE POLYMER FOR BIOMEDICAL APPLICATION

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ABSTRACT

Synthetic polymers have been widely used in biomedical applications especially in the tissue engineering field. Various kinds of polymers such as polylactic acid, polyglycolic acid, polyethylene glycol polylactic-co-glycolic acid, and polypyrrole have been explored and utilized in biomedical applications. Most of them have been used as the scaffold for tissue reconstruction except polypyrrole which has been used in nerve regeneration due to its electrical conductiveness. However, this distinct conductive property of polypyrrole polymer may replace the traditional cell harvesting technique by electrically repelling cells from the polymer substrate. Therefore, this research was conducted to investigate the significance of electrical potential that may attend to the cell growth and repulsion from the conductive polymer substrate. The substrates were constructed by electrochemical deposition method and fibroblasts were cultured on the conductive substrate. The electrical potential was applied to the substrate to repel cells. The results showed that the thickness and the surface morphology of the polypyrrole substrate can be varied by applying different voltages and currents. Moreover, fibroblasts can grow and proliferate on the substrate and can be repelled from the conductive polymer when electrical potential is applied. The repellent at -1 volt of electrical potential for 30 minutes demonstrates the best condition for repulsion in terms of high number and percent viability of repelled cells. Fibroblasts can be repelled from conductive polymer due to the electrostatic repulsion but the number and viability of repelled cells are still so low that the technique needs further improvement.

KEY WORDS: POLYPYRROLE/CONDUCTIVE POLYMER /CELL CULTURE
/REPELLENT/ELECTROSTATIC REPULSION/
FIBROBLAST

61 PP.

การศึกษาการใช้กระแสไฟฟ้าลอกเซลล์จากโพลิเมอร์นำไฟฟ้าเพื่อนำไปใช้ในทางชีวการแพทย์
(A STUDY OF ELECTRICAL CELL REPELLENT FROM CONDUCTIVE POLYMER FOR BIOMEDICAL APPLICATION)

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บทคัดย่อ

โพลิเมอร์สังเคราะห์ถูกนำไปใช้อย่างกว้างขวางในทางการแพทย์โดยเฉพาะการเพาะเลี้ยงเนื้อเยื่อ โพลิเมอร์หลายชนิดถูกสำรวจและใช้ในทางการแพทย์ เช่น โพลิแลคติดแอซิด, โพลิไกลโคลิคแอซิด, โพลิเอทิลีนไกลคอล, โพลิแลคติคโคไกลโคลิคแอซิด และ โพลิไพโรล โดยส่วนใหญ่จะถูกใช้เป็นเยื่อโครงสร้างสำหรับการซ่อมแซมเนื้อเยื่อยกเว้นโพลิไพโรลซึ่งใช้เป็นวัสดุซ่อมแซมเซลล์ประสาทเพราะมีคุณสมบัติการนำไฟฟ้า อย่างไรก็ตามคุณสมบัติที่โดดเด่นด้านการนำไฟฟ้าของโพลิเมอร์ชนิดนี้อาจนำไปใช้แทนที่เทคนิคการเพาะเลี้ยงเนื้อเยื่อตามปกติ โดยใช้กระแสไฟฟ้าลอกเซลล์จากวัสดุโพลิเมอร์ ดังนั้นงานวิจัยนี้จึงได้จัดทำขึ้นเพื่อศึกษาการใช้สนามไฟฟ้า ซึ่งอาจมีผลต่อการเจริญเติบโตของเซลล์และการลอกเซลล์จากวัสดุโพลิเมอร์นำไฟฟ้า วัสดุรองรับโพลิเมอร์นำไฟฟ้านี้ถูกสร้างโดยวิธีอีเล็คโทรเคมิคอลเดพโพสิชั่น และ ใส่ไฟโบรบลาสลงไปบนวัสดุนำไฟฟ้านี้ จากนั้นศักดิ์ไฟฟ้าถูกป้อนลงไปที่วัสดุเพื่อที่จะลอกเซลล์ ผลการทดลองแสดงว่าความหนาและลักษณะพื้นผิวของโพลิไพโรลสามารถเปลี่ยนแปลงได้ขึ้นอยู่กับศักดิ์ไฟฟ้าและกระแสไฟฟ้าที่ป้อน ไฟโบรบลาสสามารถโตและเจริญอยู่บนวัสดุได้และทำให้หลุดออกมาจากโพลิเมอร์นำ ไฟฟ้าได้เมื่อป้อนสนามไฟฟ้าเข้าไป การลอกเซลล์โดยใช้ศักดิ์ไฟฟ้า -1 โวลต์ เป็นเวลา 30 นาที เป็นสภาวะที่ดีที่สุดในการลอกเซลล์ซึ่งให้ปริมาณของเซลล์และร้อยละการรอดชีวิตของเซลล์มากกว่าทุกตัวอย่าง แม้ว่าไฟโบรบลาสสามารถลอกออกจากโพลิเมอร์นำไฟฟ้าเนื่องจากแรงผลัก อีเล็คโทรสเตติกแต่ปริมาณและการรอดชีวิตของเซลล์ต่ำมากดังนั้นเทคนิคนี้ต้องมีการปรับปรุงในภายอนาคต

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LIST OF SYMBOLS AND ABBREVIATIONS

%	Percent
/	Per
ºC	Degree Celsius
Ag	Silver
AgCl	Silver chloride
cm	Centimeter
DC	Direct current
DNA	Deoxyribonucleic acid
ECM	Extra cellular metrix
etc.	Et cetera (Latin), and so fort
$FeCl_3$	Ferric chloride
FTO	Fluorind doped tin oxide
ID	Identity
L	Litre
M	Molar
mA	Milliampere
ml	Milliliter
mmol	Millimole
nm	Nanometer
nN	Nanonewton
PEI	Polyethyleneimine
PIPAAm	Poly(N-isopropyacrylamide)
PPI	Polypropyleneimine
PPy	Polypyrrole
RPM	Round per minute
RPMI	Roswell park memorial institute
S	Seimens
sec	Second

LIST OF SYMBOLS AND ABBREVIATIONS (continued)

SEM	Scanning electron microscope
TCPS	Tissue culture polystyrene
V	Volt

CHAPTER I
INTRODUCTION

1.1 Background

Biomaterials are widely used in medical and dental treatments, although their effectiveness is questionable. Almost all biomaterials were developed for general use. Some metals and ceramics were introduced in orthopedic and dental fields and satisfactory results were obtained. Unfortunately, metallic and ceramic biomaterials are not suitable to replace soft tissues because of markedly different mechanical properties therefore the synthetic polymer become attractive biomaterial used in biomedical application. Conventional polymers are used for many of today's disposable medical devices moreover, polymers are the most commonly used in tissue engineering for organ reconstruction as a three dimensional scaffold for tissue ingrowth. Tissue engineering is an interdisciplinary field that combines with the knowledge of engineering and life science for improving tissue function [1]. This concept is investigated in the pig by implanting the mouse tumor cells seeded in the polymer scaffold and these cells are not destroyed by immune response [2].

Polymeric materials used in biomedical application have been studied including poly-α-hydroxy esters, polydioxanone, propylene fumarate, poly-ethylene glycol, poly-orthoesters, polyanhydrides and polyurethanes, poly-L-lactic acid, poly-glycolic acid, poly-L- lactic acid, and poly lactic-co-glycolic acid. These materials had already been approved for human uses and can be constructed with various porosities and 3-dimansional shape. They also showed to be an excellent substrate for cellular or bioactive molecule delivery [3, 4].

The next generation of implantable biomaterials will be interactive and programmable which enhance the communication of surrounding tissue. Specifically, materials that incorporate stimulatory cues such as electrical signals can be applied to regulate cell proliferation, differentiation and adhesion. For example, electrical field

has been demonstrated to stimulate healing of bone [5], cartilage [6], skin and connective tissue [7]; therefore many studies have been focused on the incorporation of electrical signal directly to the biomaterials especially on the conductive materials in order to stimulate cell adhesion, proliferation, differentiation and promote the organ healing.

In spite of these desirable results, polymers have been processed to display permanent charges (electrets) or to generate transient surface charges (piezoelectric materials). These electroactive materials demonstrated the enhancement of neural cell and osteocyte growth in vivo and vitro [8]. The other type of electroactive polymer is the electrically conducting polymers including polypyrrole and polythiophene. In contrast to electrets and piezoelectric materials, polypyrrole and polythiophene generate electrical signals by electron transfer between different polymer chains. They allow external control over the amount and duration of electrical stimulation, which is beneficial for biomedical applications. Moreover, conducting polymers do not require extensive processing to rendering their electroactive. These materials can also be modified with negatively charged dopant ions, which can be tailored to specific applications. For example, polypyrrole has been doped with biological anions such as hyaluronan, which stimulates angiogenesis as it degrades and adhesive peptides, which enhance material-cell interactions [9, 10].

As mention earlier, the utilization of electrical stimulation to improve the cell adhesion and proliferation can directly applied to the tissue engineering applications, however, the other fold of the electrical stimulation i.e. electrical repellent, can also applied to the cell biology as well. According to the surface chemistry, many biological cells can be forced by the electric field toward one surface having on opposite charge resulting in cell adhesion on the surface. However, by applying the opposite force field across the surface the attached cell can then be repelled from the surface which could be applied to the tissue engineering to produce a cell sheet or could be replaced the traditional cell harvesting by trypsinization. Therefore, this research is conducted in order to investigate the use of electrical stimulation to repel cell from a surface.

1.2 Objective

The goal of this research is to study the effect of electrical potential across the selected cells on conductive polymer substrate in order to investigate the potential of using this technique to harvest the cell by replacing the traditional trypsinization process.

1.3 Scope of studies

This study composes of three major tasks

(i) The preparation of conductive substrate using conductive polymer by electrochemical deposition technique.

(ii) The cell growth and cell repellant from the substrate by the aid of electrical stimulation.

(iii) The functionality of the cell after electrical treatment.

CHAPTER II
LITERATURE REVIEW

In the study on "A study of electrical cell repellent from conductive polymer for biomedical application", this chapter will present the relevant information and theories with importance on the repellent study.

2.1 Conductive polymer

2.1.1 Overview

A conductive polymer is an organic dielectric polymer or semiconductor that process electrical conductive property when it dramatically changes in its chemical nature. There are two classes; the charge transfer complexes and the conductive polyacetylenes. The latter includes polyacetylene itself as well as polypyrrole, polyaniline, and their derivatives. Conductive organic polymers often have extended delocalized bonds which composed of aromatic units that create a band structure similar to silicon but with localized states. When charge carriers from the addition or removal of electrons are introduced into the conduction or valence bands the electrical conductivity increases dramatically. However, conductive polymers generally exhibit very low conductivities**.** The interesting property of conductive polymers is their processibility. Conductive polymers are also plastics which are organic polymers and therefore can combine the mechanical properties such as flexibility, toughness, elasticity of plastics with the high electrical conductivities of a doped conjugated polymer

Conductive polymers are of interested by many researchers from a variety of fields in science and engineering as a promising electrode for energy storage device, electrochromic displays, information memory, anti-static materials, anti-corrosives, electrocatalysis, sensors, electromechanical devices, infra-red polarizers and radar [11,12]. Biomedical applications have also been considered including biosensors [13]

and the cell growth supporting substrates. Furthermore, the discovery of attraction physiological roles for in vivo electric fields as created by cells layer in order to provide wound healing, conducting polymers offer new advantages as biomaterials.

Certainly, it has been studied that small electrical currents can stimulate tissue responses such as bone re-growth, wound healing [14-16]. These were achieved using metallic electrodes inherently incompatible with biological tissues. In particular, certain tissues such as those of the nervous system [17] or skeletal and smooth muscle [18] may be particularly susceptible to modulation via electrical stimulation. Therefore, polypyrrole has become attractive for theses applications.

2.1.2 Polypyrrole

Pyrrole is one of the classes of heterocyclic compound, five-membered diunsaturated ring structure, with five carbon atoms and one nitrogen atom (figure 2.1). Polypyrrole is colorless to pale yellow, toxic oil with pungent taste and similar to chloroform odor, insoluble in water, soluble in alcohol, ether and dilute acid, boils at 129-131 °C, polymerizes by photocatalytic reaction [19].

Figure 2.1 Pyrrole ring [19].

Polypyrrole (PPy) is a chemical compound formed from a number of connected pyrrole ring structures (figure 2.2). For example a tetrapyrrole is a compound with four pyrrole rings connected. Polypyrroles are conducting polymers of the rigid-rod polymer host family. Polypyrroles are also called pyrrole blacks or polypyrrole blacks [20].

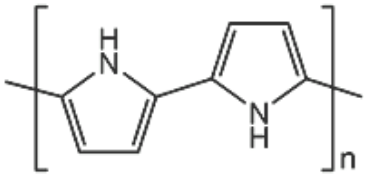


Figure 2.2 Polypyrrole [20].

2.1.3 Polypyrrole substrate fabrication

There are 2 general polypyrrole fabrication methods, chemical synthesis and electrochemical synthesis. Chemical synthesis method is employed when the large amounts of material are required and it involves with the mixing of strong oxidizing agent, particularly $FeCl_3$, with a monomer solution [21]. The electrochemical is a method preferred for the research purposes due to the simplicity of the preparation. Typically, the thickness, geometry and location, facility for doping can be controlled during electrochemical synthesis, the wide choice of dopant ions are available. Resulting in the formation of good quality substrates [22,23]. The electrochemical deposition on the positively working electrode occurs by a condensation reaction between the five-membered diunsaturated ring of pyrrole. In the electrochemical polymerization process of polypyrrole, monomer units are adsorbed onto the surface of the working electrode resulting in one-electron oxidation to form a pyrrole cation radical. These cations then couple with themselves, with other cations or with neutral monomers from solution. In each case, this leads to the formation of a dimer dication, which undergoes a double deprotonation to give a neutral molecule. These more stable dimer radicals have a lower oxidation potential compared with the monomer units and chain growth then occurs by preferential coupling between the dimers and monomers, anion (A^-) is required to maintain electroneutrality [24]. Figure 2.3 demonstrate the electrochemical polymerization mechanism of polypyrrole. Since the positive charges are developed along the polypyrrole backbone, negatively charged counterions must be added in solution in order to maintain charge balance within the polymer solution. The counterion types affect to the polymer properties. Table 2.1 summarises the parameters of electrochemical polymerization of pyrrole in the present of various dopant [25]. The doping level for PPy (number of anions per

monomer unit), depending on the nature of anions and conditions of synthesis, varies within 0.1 to 0.5. Switch-off of the current in the course of polypyrrole electrosynthesis is immediately followed by the arrest of the chain growth; the mass of the polymer film is directly proportional to the charge passed [26-28].

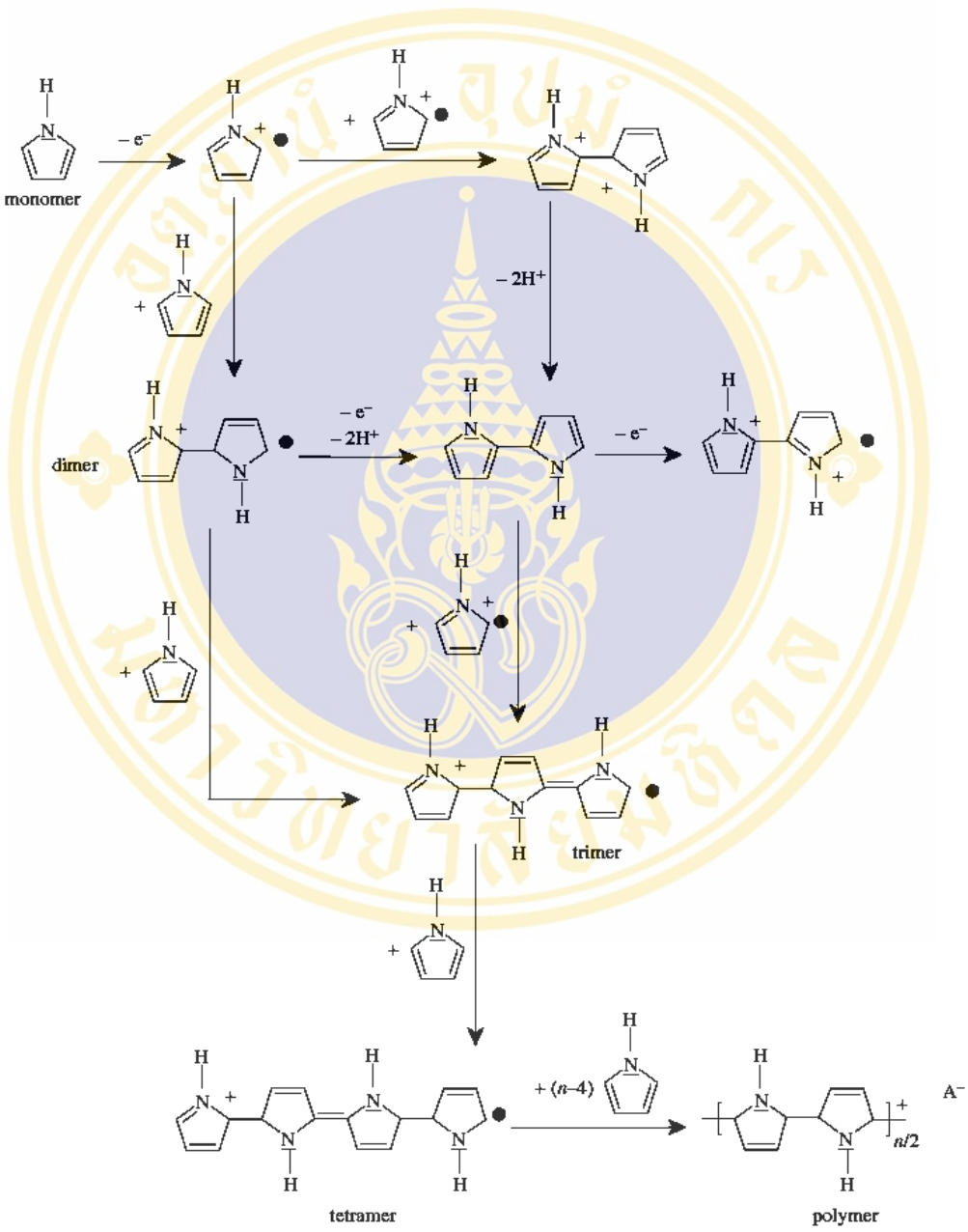


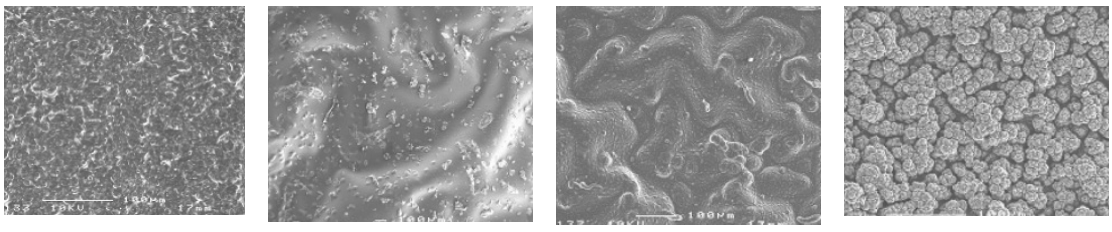
Figure 2.3 Electrochemical polymerization mechanism of polypyrrole [24].

Table 2.1 Effect of the nature of organic anion on the rate of pyrrole electrochemical polymerization and the properties of the substrate [25]

Dopant anion	Conductivity (S/cm)	Growth rate (nm/sec)
Dodecyl sulfate	15.3	1.3
Toluene sulfonate	9.8	1.0
Dodecylbenzene sulfonate	4.7	1.2
Poly (*p*-styrene sulfonate)	7.3	0.4

2.1.4 The surface morphology of polypyrrole

There are a large number of the studies for the preparations of polypyrrole each of which significantly modify the phenomenological properties of the polymer. Normally, electrochemical polymerization is carried out at the potential above 0.6 volt versus Ag/AgCl reference electrode. The morphology of substrate depends on many factors such as the nature of the dopant, the concentration of the original monomer solution, crystallographic structure of the underlying anode, the kinetics of the process and the potential used for deposition [29]. The electrochemical polymerization duration also affects both of surface morphology and thickness of substrate. The shorter times produced thin substrate with a smoother surface whereas at extended times, the substrate is thicker with distinct topography [24]. Figure 2.4 shows the morphology of polypyrrole fabricated using various dopants.



(a) (b) (c) (d)

Figure 2.4 SEM micrographs of polypyrrole doped with different counterions (a) chloride, (b) polyvinyl sulphate, (c) dermatan and (d) collagen [24].

2.1.5 The conductivity of polypyrrole

The conductivity process of polypyrrole happens in the oxidation state, bipolarons, the combination between cation radical and the local deformation of the polypyrrole backbone, migrate along the conjugated polymer chain and provide the main charge transport mechanism within the conductive polymer [23]. The conductivity of polypyrrole correlates with many factors such as dopant (mentioned earlier), and temperature. During the construction process, temperature is effect on the conductivity of polypyrrole conducting polymer films prepared by and electrochemical method. It was found that by increasing temperature resulting in the decreasing of conductivity (figure 2.5) and the optimum temperature was found to be between 10 and 30 ℃. This result shows that the polymer fabricated at low temperature has higher conductivity and is stronger than that formed at higher temperature [30].

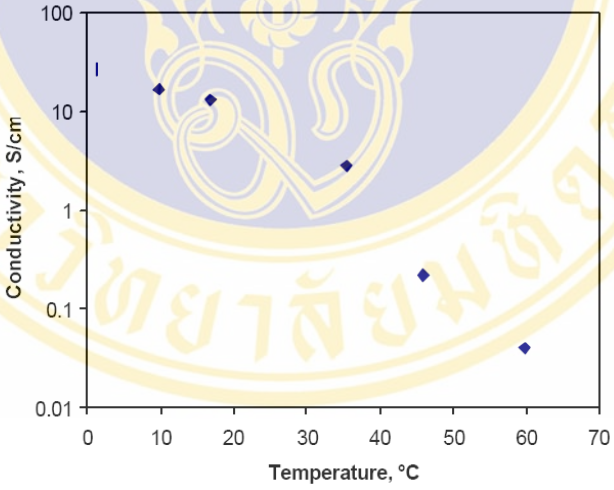


Figure 2.5 The effect of temperature on the conductivity of polypyrrole [30].

2.1.6 The modification of polypyrrole

The modification of conductive polypyrrole was done for improving some disadvantage properties of polypyrrole. For example, in order to improve the mechanical properties of the conducting polymer (e.g. brittleness), polypyrrole is blended with the other polymer. A combination of conventional polymer or copolymer with conductive polymer allows the creation of new polymeric materials

with interesting electrical properties. The chemical modification method was used for the preparation of highly conductive polymer composites of poly(methyl methacrylate) and polypyrrole resulting in a network-like structure of polypyrrole embedded in the insulating polymer matrix (31). Moreover, the non-degradation property of polypyrrole was solved by blending with polylactide. The biodegradable material made of polypyrrole and polylactide was prepared by emulsion polymerization of pyrrole in a polylactide solution, followed by precipitation. Polypyrrole particles formed aggregations and constituted microdomains and networks embedded in the polylactide. the study showed that 1–17% increased in the polypyrrole content, the conductivity of the composite increased by six orders of magnitude (figure 2.6) [32].

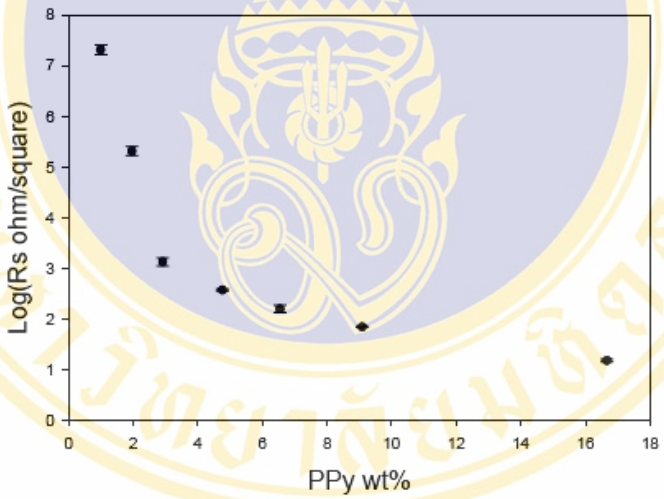


Figure 2.6 The relationship between the surface electrical resistivity of the polypyrrole/polylactide membranes and the polypyrrole content [32].

The other modification of polypyrrole is done to create the biodegradable conducting polymer. Ester linkage which can be cleaved by enzyme such as chllesterol esterase was inserted to the pyrrole-thiophene oligomers using aliphatic linker [33].

2.1.7 Polypyrrole in medical application

It was found the electrical stimulation that electrical stimulation is capable of modifying cellular activities such as cell migration [34], cell adhesion [35], DNA synthesis [36] and protein secretion [37]. These make electrical stimulation potentially enhance the regeneration of damaged tissues and make polypyrrole becomes more attracted to many groups of researcher. Since in early nineties, polypyrrole has been substantially studied as a cell growth substrate within *in vitro* culture. Moreover, the effects of implantation *in vivo* have also been studied using animal model. Most of polypyrrole used in the research was constructed using electrochemical deposition method because of the reason mentioned previously.

Garner et. al., studied on the culture of human umbilical vein endothelial cells on polypyrrole doped with heparin. They showed that the polypyrrole/heparin composite supported the growth endothelial cells [38]. The work of Collier et. al. considered in the polypyrrole doped with glycosaminoglycan, hyaluronic acid.
The *in vitro* biocompatibility studies using PC-12 cells confirmed that the polypyrrole/hyaluronic composite supported cell attachment and viability [9]. The studies of Cui et. al. focus on the neural signal recording device. Neural recording microelectrodes have been coated with polypyrrole doped with fibronectin and laminin fragments. They found that the coating polypyrrole did not interfere with recording when measurements were made in guinea pig cerebellum [10]. Williams et. al. established that polypyrrole doped with tetra-ethyl-ammonium p-toluene sulfonate exhibit a good compatibility with L292 mouse fibroblast [39]. George et. al. considered the response of rat cortical tissue both *in vitro* and *in vivo* on polypyrrole doped with various dopant. The results indicated that the favourable responses compared to Teflon implants in term of macrophage activity, gliosis and neuronal integration after implantation [40]. Ateh et. al. investigated the polypyrrole doped with the variety of incorporating proteins. These are feasible for producing coherent membranes and are able to support keratinocyte growth [41]. Wong et. al. studied the viability of bovine aortic endothelial cells on polypyrrole substrate coated with fibronectin. Further experiments, where cell cultured substrate were switched from oxidized to reduced states by applying a small negative electrical potential, revealed cellular response within an hour [42]. While the poor interaction occur between

polypyrrole (PPy) and neuronal cell compared to other materials, polyethyleneimine (PEI), polypropyleneimine (PPI) and fluorind doped tin oxide (FTO) were demonstrated by Lakard et. al. Neuronal cell line adhesion and proliferation on different substrates after 8, 24 and 72 hrs of culture. The numbers of cells were normalized to initial density of seeded cells (200,000 cells/ml) (n=3 per substrates). The volume of the cell suspension used is 100 ml. The results were shown in figure 2.7 [43].

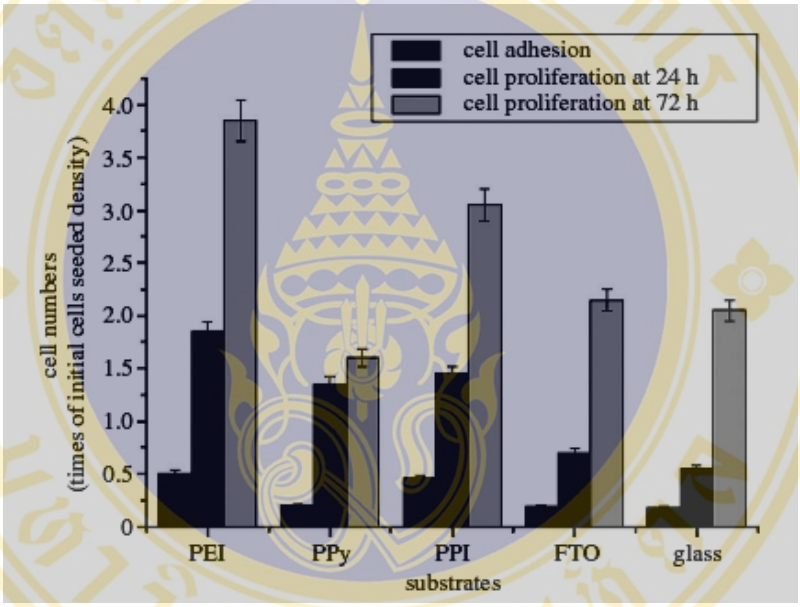
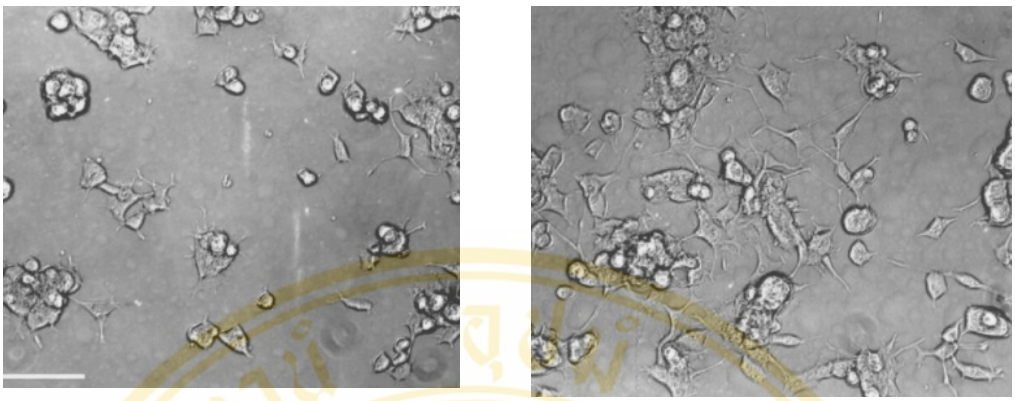


Figure 2.7 The interaction between cell numbers cultured on the various type of supporting material [43].

It has already been demonstrated that application of an electrical stimulus to PC-12 cells (a neuron-like cell line) cultured on polypyrrole significantly enhances neurite extension by 90% compared to cells grown on polypyrrole without electrical stimulation (see figure 2.8) [44]. Preliminary *in vivo* studies with polypyrrole conduits fitted around damaged nerve ends showed that these conduits physically guided the regeneration of rat sciatic nerve across a 10 mm defect significantly better than silicone controls [45].



(a) (b)

Figure 2.8. PC-12 cell differentiation on polypyrrole (a) without and (b) with application of an electric potential [45].

2.2 Electrical repulsion

2.2.1 Attractive force and repulsive force

In order for molecules to exist in aggregates in gases, liquids, or solids, there must be forces that attract the molecules together. This is a key concept in understanding the stabilization of emulsions, the compression of powders into capsules, and the attraction of the drug to the substrate in the body. When the repulsive and attractive forces are equal the net potential energy is at a minimum and the system is stable (figure 2.9) [46].

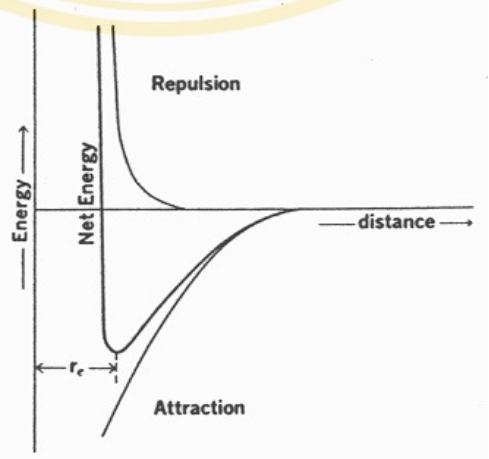


Figure 2.9 The relation between the distance of particles and energy [46].

2.2.1.1 Repulsive Forces

The force is repulsive when the molecules are brought close enough together that the outer charge clouds of the molecules touch, and this causes the molecules to repel each other. The repulsive forces are necessary so that the molecules do not destroy each other [46].

2.2.1.2 Attractive Forces

The forces that bring molecules together are called forces of attraction. These forces include cohesion, the attraction of like molecules, and adhesion, the attraction of unlike molecules. Attractive forces are divided into two groups: strong forces and weak forces. The weak forces of attraction are: van der waal's forces, ion-dipole forces, and hydrogen bonds. The strong forces include the ionic and covalent bonds [46].

2.2.2 Electrostatic force of cells

Normally, the surfaces of biological cells are negative charge due to the presence of proteins and cell membrane which consists of phosphate, carboxyl and other acidic groups. However, the living cells need to stay in the supplement fluid therefore the net surface charge is not simply as mentioned because the negative cell surface charge attract oppositely charged ions from the surrounding fluid. These ions which are attracted to the surface form a mobile layer called electric double layer (see figure 2.10) [47]

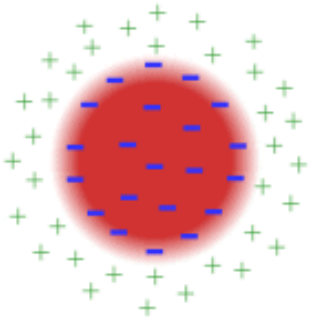


Figure 2.10 The electric double layer of cell [47].

2.2.2.1 The electrostatic repulsion force

The electrostatic repulsion force of biological cells is not easy to calculate because there are many factors that need to take into account. However, the electrical repulsion can be simplified as shown in equation 2.1 [47].

$$Force = \frac{332 q_1 q_2}{Dr} \qquad \text{Equation 2.1}$$

Where q_1 and q_2 are the total charge of cells.

D is the dielectric constant as the surrounding fluid.

r is the distance separating surfaces.

The interaction force between two charged surfaces is inversely proportional to the distance between them (see figure 2.11). The direction of force also depended on the type of surface charge of two surfaces. The repulsive force occurred when two surfaces possess the same charge, whereas the attractive force occurred when two surface process the opposite charge [47].

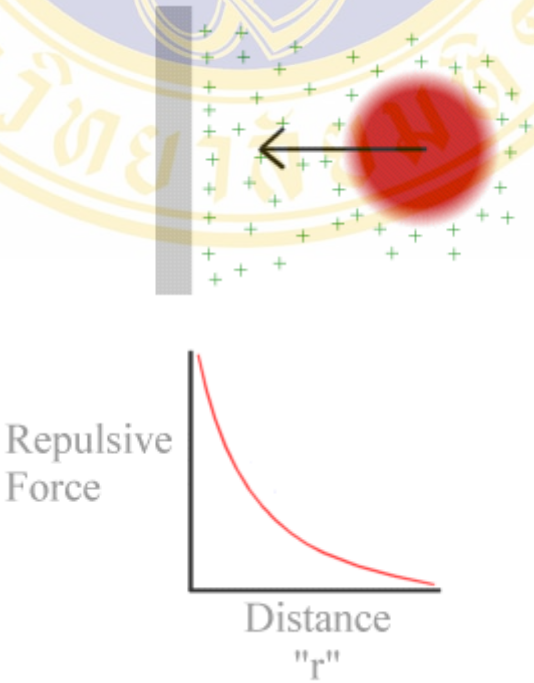


Figure 2.11 The relationship of repulsive force and the distance between 2 charges [47].

2.2.3 Adhesion force of cell

Different cell types adhere with different forces. The glass needles were used to measure cell–cell adhesion forces as large as 10,000 nN in normal cervix epithelial cells, whereas this apparatus lacked sensitivity to measure the force between cervical carcinoma cells. However, the data indicate that this force is less than 2,000 nN [48]. By using a high-speed centrifugation technique, epithelial cells were exposed to forces tangential to the substrate. These cells started to dissociate from the surface at 100 nN [49]. Recently, the adhesion forces of murine fibroblast cells were measured by using a technique employing ideas similar to the manipulation force microscope. Forces between 300 and 400 nN were necessary to remove these cells from a serum protein-covered glass substrate [50]. The traction force exerted by fibroblasts on an elastic membrane may be as large as 1,200 nN [51].

2.2.4 Electrical repulsion

The charged particles including living cells normally move from the similar charged electrode to the opposite charged electrode in the electrical field (figure 2.12).

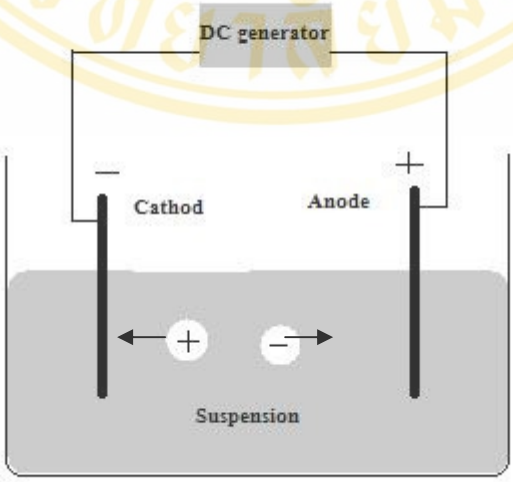


Figure 2.12 The movement of charged particles in the electrical field.

2.3 Various methods of cell repellent

There are some recent studies about the cell repellent in order to avoid the trypsinization process including the temperature responsive culture dish, human fibrin-coated dish and electrostatic repulsion of lipid/DNA complex.

2.3.1 Cell repellent by using temperature responsive culture dish

Temperature-responsive culture surface were developed among the research to control cell adhesion to biomaterial. Cells adhere to culture surface via membrane receptors and cell adhesive protein, including fibronectin that reside in serum or are secreted from the cells in culture (figure 2.13A). The interaction between adhesive proteins and culture surfaces depends on the wettability of the surface. Normal tissue culture polystyrene (TCPS) dishes are hydrophobic and absorb extra cellular matrix proteins resulting in cell attachment and proliferation. To harvest cells from the surface, enzymatic digestion including typsin and lipase are usually utilized. In that case, adhesive proteins and membrane receptors are disrupted, then cells detach with considerable damages (figure 2.13B). On the other hand, The temperature-responsive polymer, poly(N-isopropyacrylamide) (PIPAAm) is grafted to TCPS dishes covalently by electron beam. The surfaces are hydrophobic and cells adhere and proliferate under culture condition at 37 ºC. By lowering temperature below 32 ºC, the surface change reversibly to hydrophilic and cells are detached due to rapid hydration and swelling of the grafted PIPAAm. This unique surface changed allows cultured cells to detach spontaneously from these grafted surfaces simply by lowering temperature [52]. As against using enzymatic digestion, only the interaction between adhesive proteins and material surface is released and cells detach together with intact membrane protein and adhesive protein (figure 2.13C) [53]. As a result, cells recovered by temperature-response culture dish maintain their differentiated function more strongly than the cells recovered by protease digestion [54].

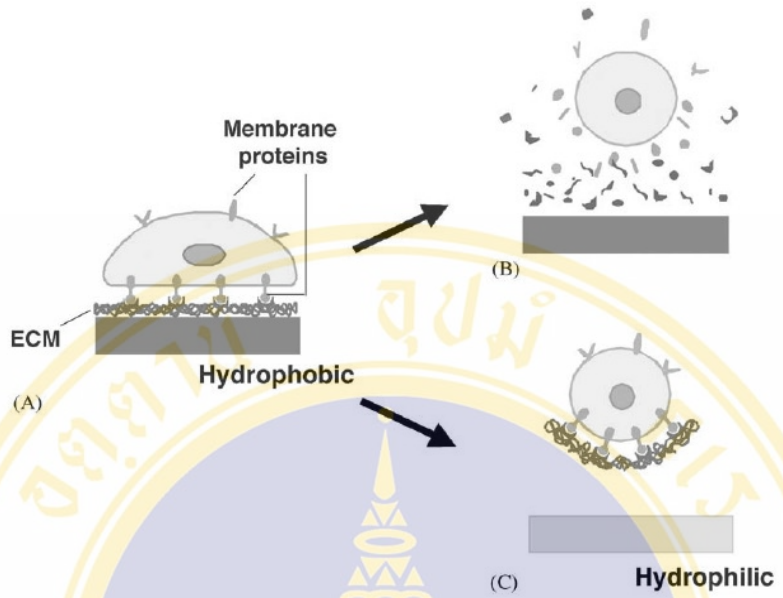


Figure 2.13 Cell harvest mechanism by using temperature-responsive culture surfaces. (A) Cells attach to hydrophobic culture surfaces via cell membrane proteins and ECM, which reside in serum or are secreted from the cells. (B) When enzymatic digestion is used, both membrane and ECM proteins are disrupted, resulting in cell detachment. (C) When cells are cultured on temperature-responsive culture surfaces; the interconnection between ECM and hydrophilic culture surfaces is released only by lowering temperature. Then the cells detach together with intact proteins [52].

2.3.2 Cell repellent using human fibrin-coated dishes

The human fibrin-coated dishes were prepared for making functional myocardial cell sheets that may be used as transplants. Polymerized human fibrin-coated dishes were prepared with fibrinogen monomers mixed with thrombin. Neonatal rat cardiomyocytes cultured on these dishes formed myocardial cell sheets within 4 days. These cell sheets were easily dissociated intact from the polymerized fibrin layer, because the fibrin had been digested by intrinsic protease (figure 2.14) [55].

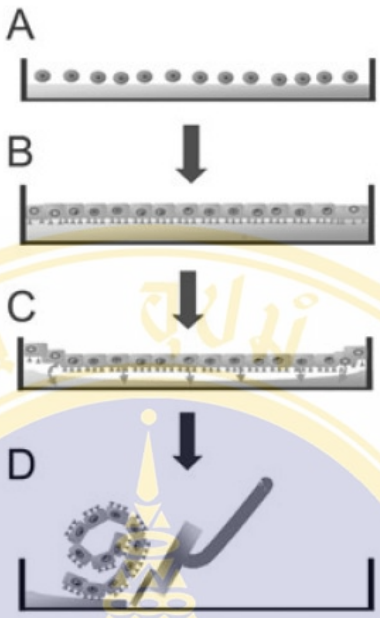


Figure 2.14 Representative schema of the manipulation of myocardial cell sheets using polymerized fibrin-coated dishes. (A) Primary cultured neonate rat cardiomyocytes were spread onto the polymerized fibrin-coated dishes. (B) Cardiomyocytes became confluent. (C) In 4 days, the fibrin polymer had been degraded by proteases secreted from cardiomyocytes. (D) Cells were gently raked from the edge toward the center of the dishes so as not to tear the myocardial cell sheets with the cell scraper [55].

2.3.3 Electrostatic repulsion of lipid/DNA complex

This study proposes the application of electrostatic repulsion in gene delivery system, to address all concerns on non-toxic, easy, and possibly efficeint delivery systems. The negatively charged lipid/DNA complexes (lipoplexes) can be electrostatically adsorbed on the gold microelectrode surface. The resulting lipoplexes molecules can be subsequently removed from the surface by applying – 1.0 volts versus Ag/AgCl reference electrode in phosphate buffer medium. The authors concluded that the release mechanism is likely due to the electrostatic repulsion between lipoplexes and the negatively charged electrode surface. The schematic drawing of the lipoplexes repellent was shown in figure 2.15 [56].

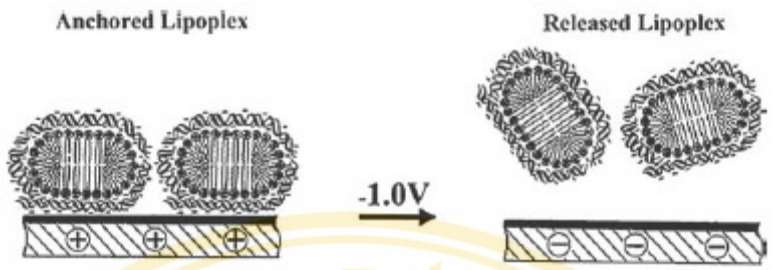


Figure 2.15 The schematic drawing representing the release of lipoplexe layers from gold microelectrode surface by applying the electrical potential [56].

CHAPTER III
MATERIALS AND METHODS

This research performs in the construction of conductive polymer, cell culturing on the substrate, the repellent of cell from conductive substrate by the aid of electric field and the functionality testing of cell after repellent in order to apply to the tissue engineering aspect. The materials and methods used in this research are shown as follow.

3.1 Materials and instruments

In this research, Materials are used to fabricate the conductive substrate and perform the experiment. The instruments are employed to characterize the substrate and execute the experiment.

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

1. Pyrrole monomer (Sigma, USA)
2. Sodium dodecyl sulfate(SDS) (Fluka, USA)
3. Absolute ethanol
4. Distilled water
5. RPMI culture media
6. Inactivated fetal calf serum
7. Trypan blue dye
8. Trypsin
9. Vitros Chemistry Products LAC Slides

3.1.2 Instruments used in this research are as follows:

1. Potentiostat/Galvanostat
2. DC generator (Model KENWOOD, PW18-IT)
3. 4X4 cm. Platinum plate
4. Platinum rod
5. Stand and clamp
6. 150 ml Beaker
7. Multimeter
8. Autoclave
9. 3.5 cm Petri dish
10. Autopipet
11. Pipet
12. Laminar flow
13. Optical microscope
14. Centrifuge
15. Scanning Electron Microscope (SEM)(Philip: XL30&EDAX)
16. Vitros Chemistry System (Vitros 250)
17. Incubator

3.2 Methods

This research studies on the fabrication of conductive polypyrrole substrate using electrochemical deposition technique. The obtained substrates were applied to the cell culturing for observing the possibility to use it in tissue engineering and using in the electrical cell repellent study.

3.2.1 The substrate fabrication using electrochemical deposition method

In this study, 0.1 M of pyrrole monomer was dissolved in absolute ethanol and mixed with 0.1 M of sodium dodecyl sulfate in distilled water. The solution was stirred using magnetic stirrer to enhance the miscibility. The 3 electrode systems which consist of platinum plate (working electrode), platinum rod (counter electrode) and Ag/AgCl (reference electrode) were immersed in this solution (see figure 3.1).

The 0.7 V, 0.8 V, 0.9 V, and 1.0 V of electrical potentials and 10 mA, 20 mA and 30 mA of electrical currents were applied to the electrode for 1, 2, 3 and 4 hour(s) respectively. After fabrication, the substrates depositing on the platinum working electrode were peeled off by a knife. The surface morphology and thickness of the substrates were observered using SEM**.**

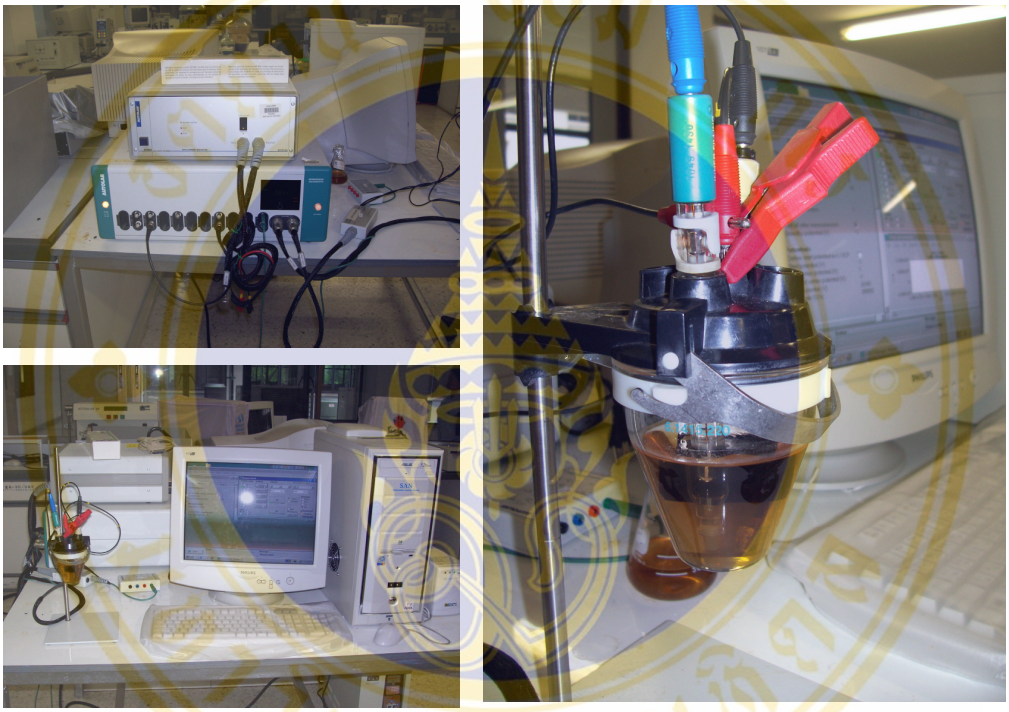


Figure 3.1 Equipments set up for the substrate fabrication.

3.2.2 Fibroblast culture on the conductive substrate

The L929 fibroblasts were obtained from malaria research laboratory, faculty of science, Mahidol University. Fibroblasts were passaged before used in order to calculate the amount of cells. Then fibroblasts were repelled from the culture flask by trysinization. The culture media of 5% fetal calf serum in RPMI was removed by pipet. The fibroblast was rinsed with 10 ml RPMI media for 5-6 times. The 3 ml of trysin were added to the culture flask and incubated in incubator of 3 minutes. The reaction was terminated by adding the 10 ml RPMI and transfer to the 50 ml sterilized tube. The fibroblast was then centrifuged at 1500 RPM for 10 minutes and the supernatant was discarded. The amounts of cells were counted using the

hemacytometer by mixing the cells suspension with trypan blue dye. The total amounts of cells were calculated by the equation 3.1.

$$N=CxDxRx10^4 \text{ Cells} \qquad \text{Equation 3.1}$$

Where N is the total amount of cells

C is the counted cells

R is the ratio of cells suspension and trypan blue dye

D is dilution factor

The 1x1 cm^2 conductive substrates were prior sterilized in autoclave and washed with RPMI for 5-6 times. The $5x10^4$ cells of fibroblasts were loaded to the substrates which placed on the 3.5 cm culture dishes and 3 ml of 5 % fetal calf serum in RPMI culture media was added to the culture dish, and incubated at 37 ^oC. Culture media was changed every 2-3 days. The growth of cells was observed continuously by using inverted light microscope. The cell viability was determined by the quantity of lactic acid secreted from the cell metabolism by the aid of Vitros Chemistry System.

3.2.3 The studied of electrical cell repellent

The experiments of electrical cells repellent were set into 3 groups of studies

(i) The variation of electrical potentials; the electrical repellent potentials were varied from -1 V, -3 V, -5 V and -7 V.

(ii) The variation of culture period; the fibroblasts culturing on the conductive substrate were collected after 7, 14 and 21 days of culturing for repulsion examination.

(iii) The variation of repelling time; the repelling times were varied from 15 to, 30, 45 and 60 minutes.

The conductive substrate with fibroblast layers was connected to the negative terminal of a DC generator while the platinum plate was connected to the ground. The simplified equipments set up was shown in figure 3.2.

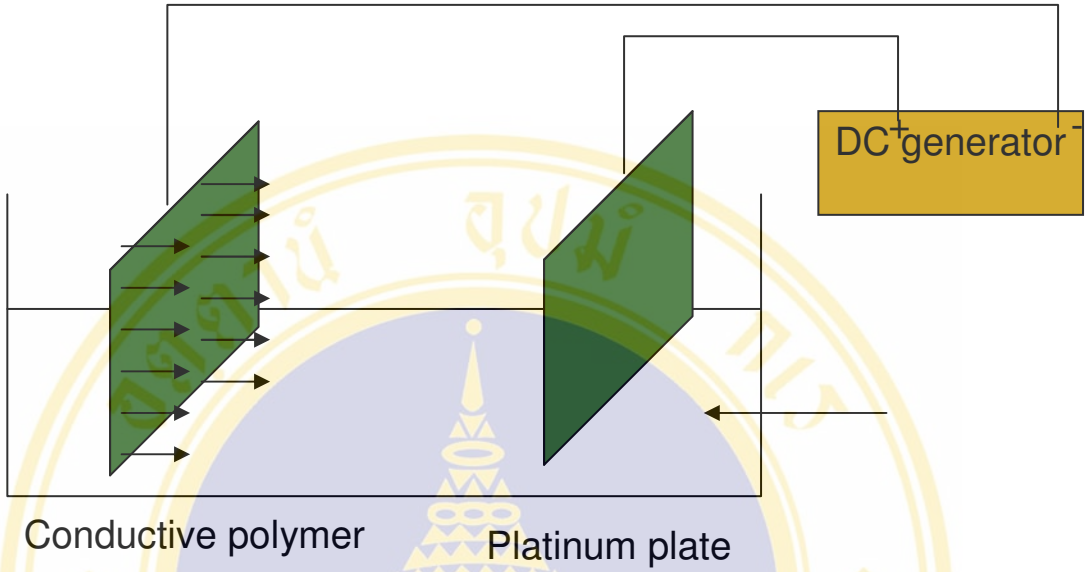


Figure 3.2 The equipments set up for the electrical cell repellent study.

CHAPTER IV
RESULTS

4.1 Substrate construction and characterization

The conductive polypyrrole substrate constructed from electrochemical deposition method gave a dark, thin, and brittle membrane. The size of substrate is limited by the size of working electrode as shown on figure 4.1

Figure 4.1 The conductive polypyrrole fabricatedd from electrochemical method.

To characterize the surface morphology and the thickness of the conductive polymer substrate, scanning electron microscope was employed for the examination. Electrical potential, electrical current and construction time were varied for the substrate construction.

4.1.1 Polypyrrole substrates constructed from a constant electrical potential applied.

Surface morphology of conductive polypyrrole constructed at a constant potential of 0.7, 0.8, 0.9 and 1.0 volt for 1 hour was observed using SEM. All substrates constructed by constant electrical potential showed the high roughness surface. The results were shown in figure 4.2

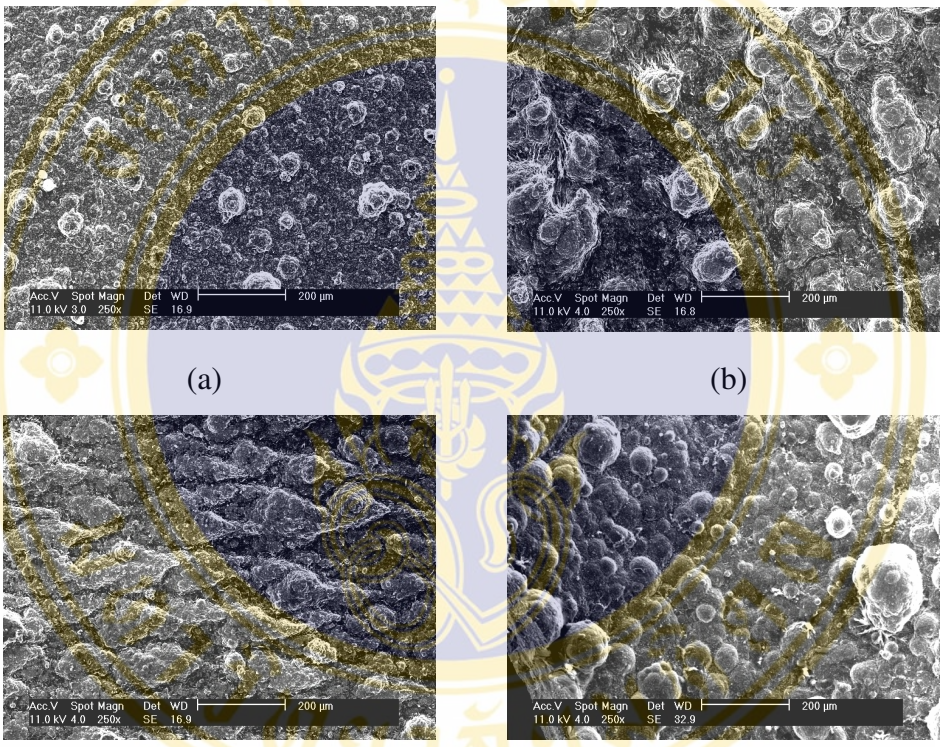
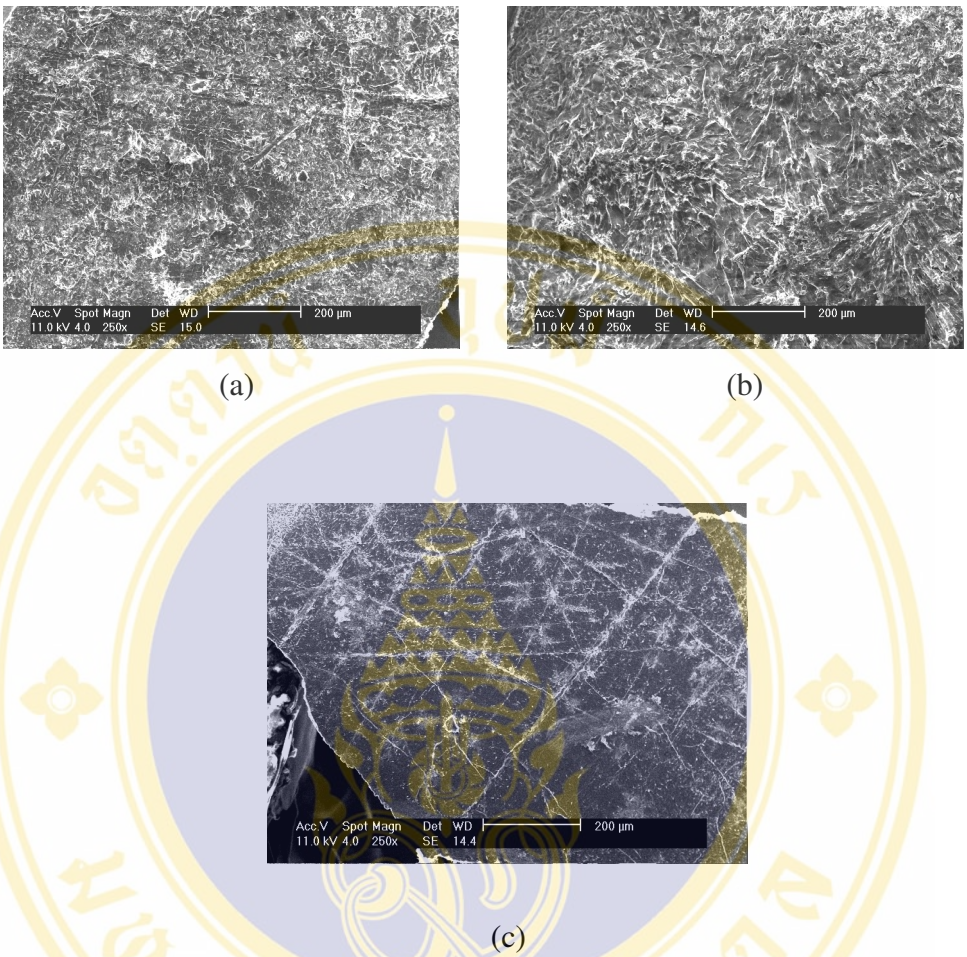


Figure 4.2 The effect of electrical potential on the surface morphology of conductive polypyrrole fabricated at (a) 0.7 volt, (b) 0.8 volt, (c) 0.9 volt and (d) 1.0 volt for 1 hour.

Whereas the SEM results of the surfaces which contact to the working electrode, referred to back side, constructed from 0.8 volt, 0.9 volt and 1.0 volt showed the smoother surface than the side facing to the counter electrode. However, In comparison with each others, the surface morphologies of these back sides of polypyrrole substrates were the same. The SEM micrographs were shown in figure 4.3.



(a) (b)

(c)

Figure 4.3 The effect of electrical potential on the surface morphology of conductive polypyrrole (back side) fabricated at (a) 0.8 volt, (b) 0.9 volt and (c) 1.0 volt for 1 hour.

The thickness of conductive polypyrrole substrates constructed at vatious electrical potential for 1 hour at 0.7, 0.8, 0.9 and 1.0 volt, were measured using SEM. The results shows that the thickness of polypyrrole substrates constructed at a variety of electrical potential were proportional to the amount electrical potential. The higher the electrical potential used, the thicker the substrate was. The results were shown in table 4.1.

Table 4.1 The effect of electrical potential in the thickness of conductive substrate.

Sample ID	Applying voltage	Fabrication time	Substrate thickness
07V1H	0.7 volt	1 hour	50-60 µm
08V1H	0.8 volt	1 hour	70-80 µm
09V1H	0.9 volt	1 hour	90-100 µm
10V1H	1.0 volt	1 hour	100-110 µm

The effect of fabrication period to the thickness of conductive substrate constructed at 0.9 volt for 1, 2, 3 and 4 hour(s) were measured using SEM. The results showed that the thickness of polypyrrole substrates were proportional to the time of construction. At a longer period of fabrication, the thicknesses of conductive polypyrrole substrates were increased . The results were shown in table 4.2.

Table 4.2 The effect of fabrication time in the thickness of conductive substrate fabricated at a various electrical potential.

Sample ID	Applying voltage	Fabrication time	Substrate thickness
09V1H	0.9 volt	1 hour	90-100 µm
09V2H	0.9 volt	2 hours	100-110 µm
09V3H	0.9 volt	3 hours	110-120 µm
09V4H	0.9 volt	4 hours	160-170 µm

4.1.2 Polypyrrole substrates constructed from a constant electrical current applied.

Surface morphology of conductive polypyrrole substrates constructed from the constant electrical currents were characterized using SEM. The constant 10 mA, 20 mA and 30 mA of electrical currents were chosen to fabricate the conductive substrates. The SEM micrographs of polypyrrole substrates showed the moderate roughness of the substrate surfaces. This surface morphology of the substrates fabricated from electrical current applied is smoother than those fabricated from a constant electrical potential. The results were shown in figure 4.4.

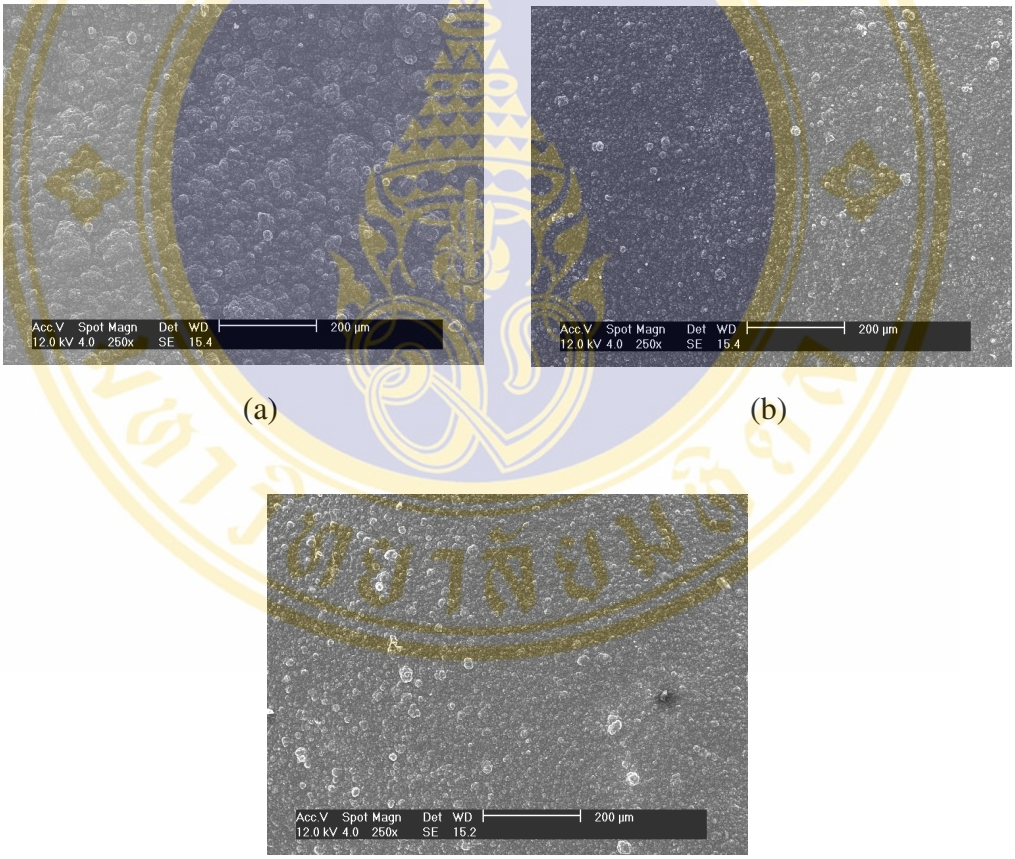


Figure 4.4 Effect of electrical current on the surface morphology of conductive polypyrrole fabricated at (a) 10 mA, (b) 20 mA and (c) 30 mA for 1 hour.

The SEM results of the back side of conductive polypyrrole substrates constructed from 10 mA, 20 mA and 30 mA showed the smoother surface than the front. However, In comparison with each others, the surface morphologies of these back sides of polypyrrole substrates were the same. The SEM micrographs of back side of polypyrrole substrates fabricated from various electrical currents were shown figure 4.5.

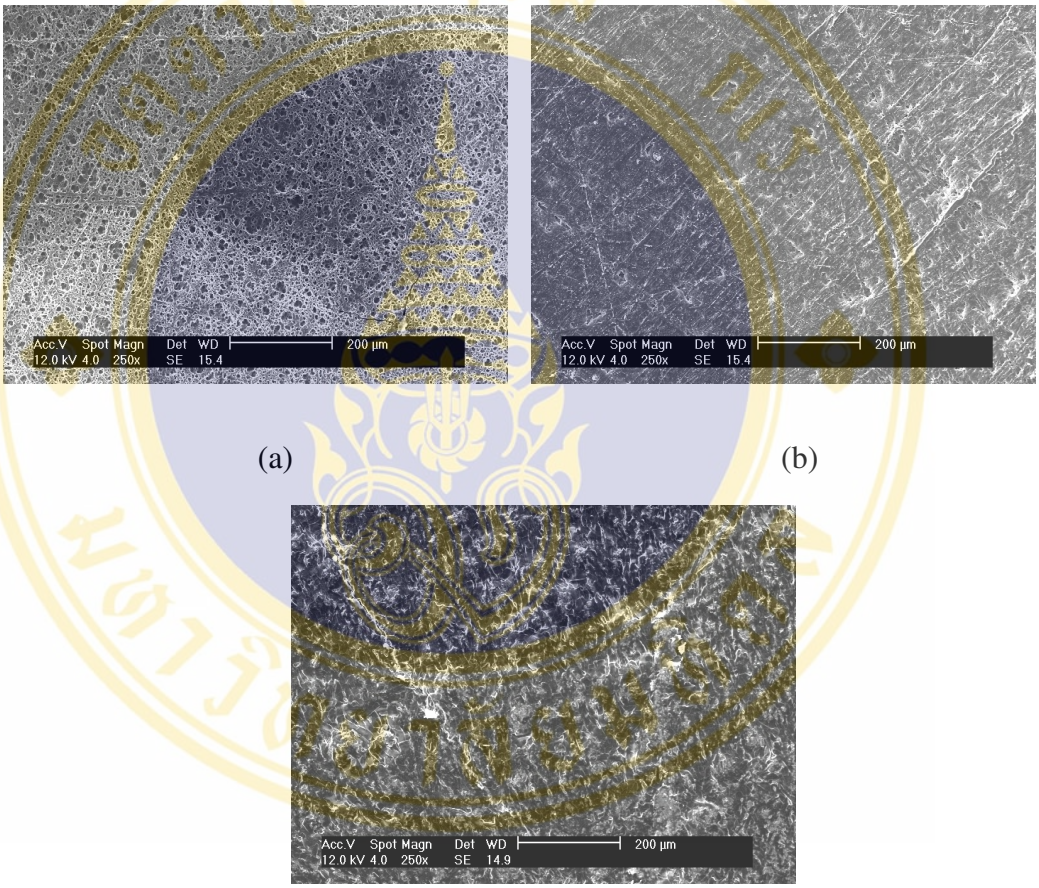


Figure 4.5 The effect of electrical current on the back surface morphology of conductive polypyrrole (back side) constructed at (a) 10 mA, (b) 20 mA and (c) 30 mA for 1 hour.

The thickness of conductive polypyrrole substrates constructed at constant electrical current for 1 hour at 10 mA, 20 mA and 30 mA, were measured using SEM. The results shows that the thickness of polypyrrole substrates constructed at a variety

of electrical currents were proportional to the amplitude of electrical current applied. The higher the electrical current applied, the thicker the substrate was. The results were shown in table 4.3.

Table 4.3 The effect of electrical currents in the thickness of conductive substrate.

Sample ID	Applying current	Fabrication time	Substrate thickness
10A1H	10 mA	1 hour	5-10 µm
20A1H	20 mA	1 hour	15-20 µm
30A1H	30 mA	1 hour	180-185 µm

The effect of fabrication period to the thickness of conductive substrate constructed at 10 mA for 1, 2, 3 and 4 hour(s) were observed using SEM. The results showed that the longer period of electrical current applied to the electrode, The thicker the substrate was. The result were shown in table 4.4

Table 4.4 The effect of construction time in the thickness of conductive substrate fabricated at various electrical currents.

Sample ID	Applying current	Fabrication time	Substrate thickness
10A1H	10 mA	1 hour	5-10 µm
10A2H	10 mA	2 hours	50-60 µm
10A3H	10 mA	3 hours	60-70 µm
10A4H	10 mA	4 hours	150-160 µm

4.2 Cell culture on the conductive substrate

The 5 x 10^5 of fibroblast cells were loaded to the 1x1 cm of conductive substrate. After 5 days of culturing, the substrates were placed to the new culture dishes. The cells growth and cells proliferation were observed continuously using inverted light microscope and lactic acid test.

4.2.1 Optical microscopic examination

The microscopic results of fibroblast growing on the conductive substrate after 2,7,9 and 14 days of culturing were shown in figure 4.6

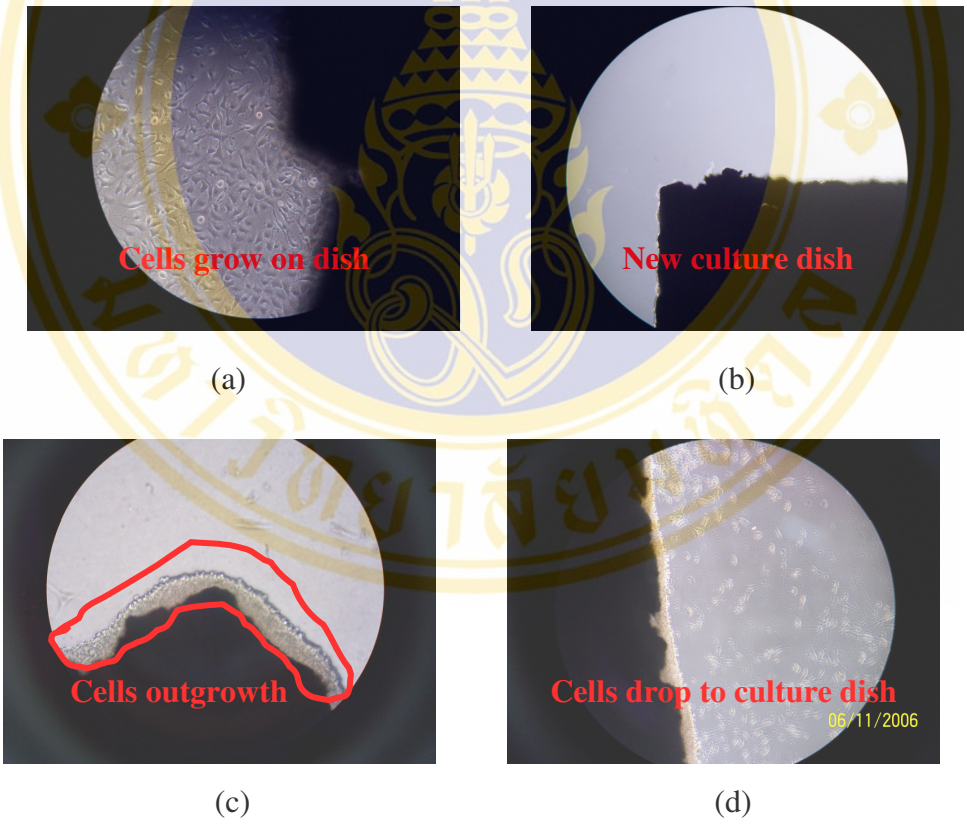


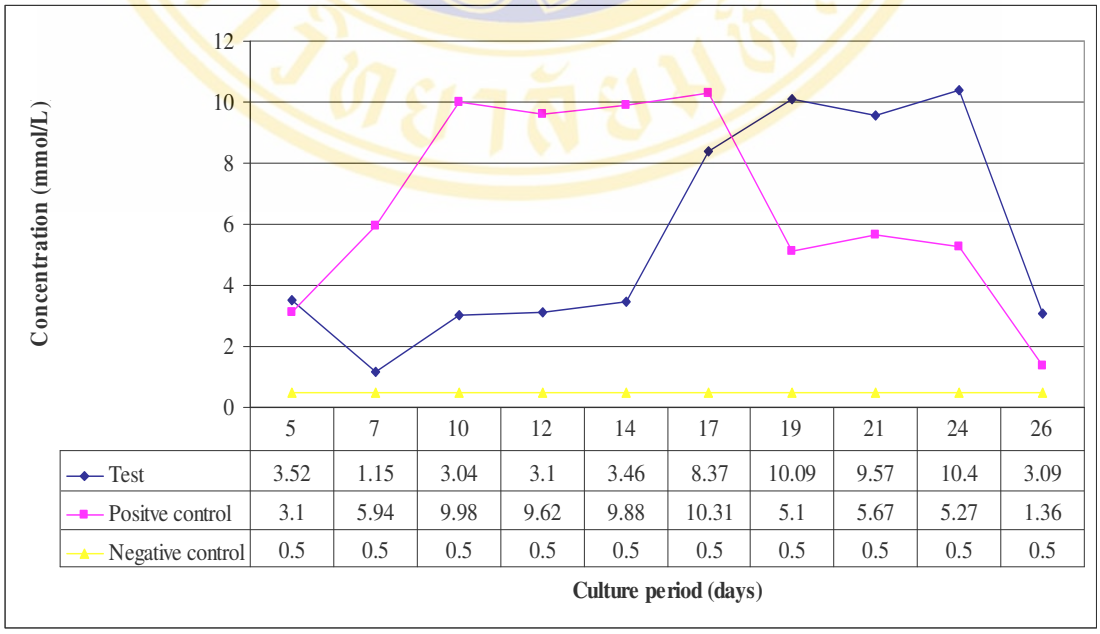
Figure 4.6 The micrographs of fibroblast growing on the conductive substrate at (a) 2, (b) 7,(c) 9 and (d) 14 days of culturing.

After 2 days of culturing, fibroblasts were found on the surface of culture dish. The conductive substrates were then removed and placed onto a new culture dish at

5days of culturing in order to avoid the error of lactic acid secreted from the fibroblasts growing on the culture dish. The cell growth on the substrate was continuously observed throughout the entire period of study. Although, the image taken after 7 days of culturing did not show any fibroblast cell proliferate to the edge of the substrate. The image taken after 9 days of culturing showed the dense layer of the cells around the perimeter of the substrate. This cell proliferation was continued until the fibroblasts spreaded out to the surface of culture dish at 14 days of culturing. of culturing.

4.2.2 Lactic acid examination

The lactic acid concentration test was used for the fibroblast growth examination. The $5x10^5$ fibroblasts were loaded to the 1x1 cm^2 of substrate and the culture dish acts as a positive control while the substrate without fibroblast loading was used as a negative control. After 5 days of culturing, the substrate with fibroblasts was placed to the new culture dish in order to avoid the error from the fibroblasts adhering on the culture dish. The lactic acid concentration of fibroblasts growing on the conductive polypyrrole substrate was analyzed and shown in figure 4.7.



	5	7	10	12	14	17	19	21	24	26
Test	3.52	1.15	3.04	3.1	3.46	8.37	10.09	9.57	10.4	3.09
Positve control	3.1	5.94	9.98	9.62	9.88	10.31	5.1	5.67	5.27	1.36
Negative control	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5

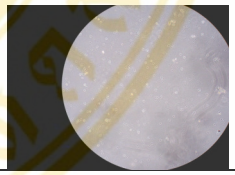
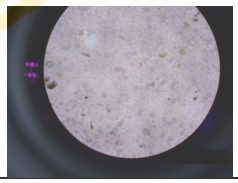
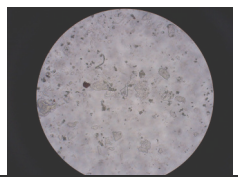
Figure 4.7 The lactic acid concentration of fibroblast culturing on the conductive polypyrrole substrate at various culture period.

4.3 Electrical cell repellent

Fibroblasts were repelled from the conductive substrate by applying the electrical potential across the substrate and counter electrode. The electrical potential, repelling time and day of culturing were varied in order to study the effect of these conditions to the repellent.

The number of cell on substrates cultured at various duration (7, 14, and 21 days) were examined using constant potential (-3 volts) for 30 minutes of repellent (see table 4.5). The number of cell and % viability of cells repelled from the conductive substrate at variety of culturing days were counted using trypan blue assay. The results were shown in figure 4.8.

Table 4.5 The results of electrical cell repellent from conductive substrate by applying -3 volts for 30 minutes at various culture period.

Sample ID	Culture period	Result
3V30M7D	7 days	
3V30M14D	14 days	
3V30M21D	21 days	

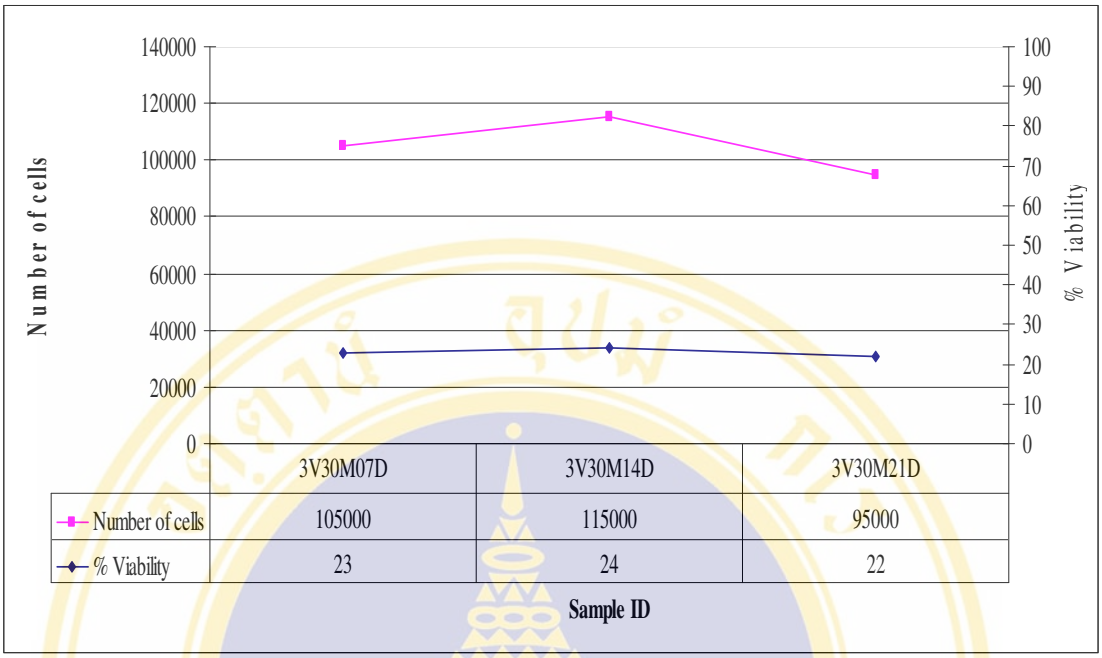
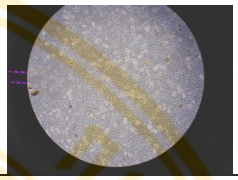
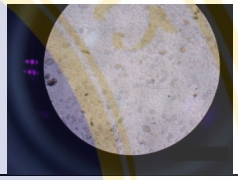
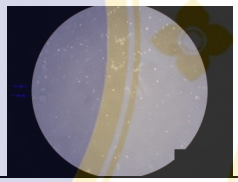


Figure 4.8 The number and % viability of cells repelled at variation of culture period.

Conductive substrates with fibroblasts were immersed in the RPMI media against the platinum plate counter electrode. The substrates cultured for 14 days were chosen for this study due to the large number of cell grown on these substrates. The electrical potential used for repelling was varied from -1, -3, -5, and -7 volt(s), the repelling period was kept constant for 30 minutes for every sample. The macroscopic results of repelling in RPMI media were shown in table 4.6.

Table 4.6 Results of electrical cell repellent from conductive substrate by applying the various electrical potential at day 14th of culturing and using 30 minutes of repelling time.

Sample ID	Applying voltage	Result
1V30M14D	-1 volt	
3V30M14D	-3 volts	
5V30M14D	-5 volts	
7V30M14D	-7 volts	

The number of cell repelled from the conductive substrate at different electrical potentials and % viability of cells were counted using trypan blue assay. The results were shown in figure 4.9.

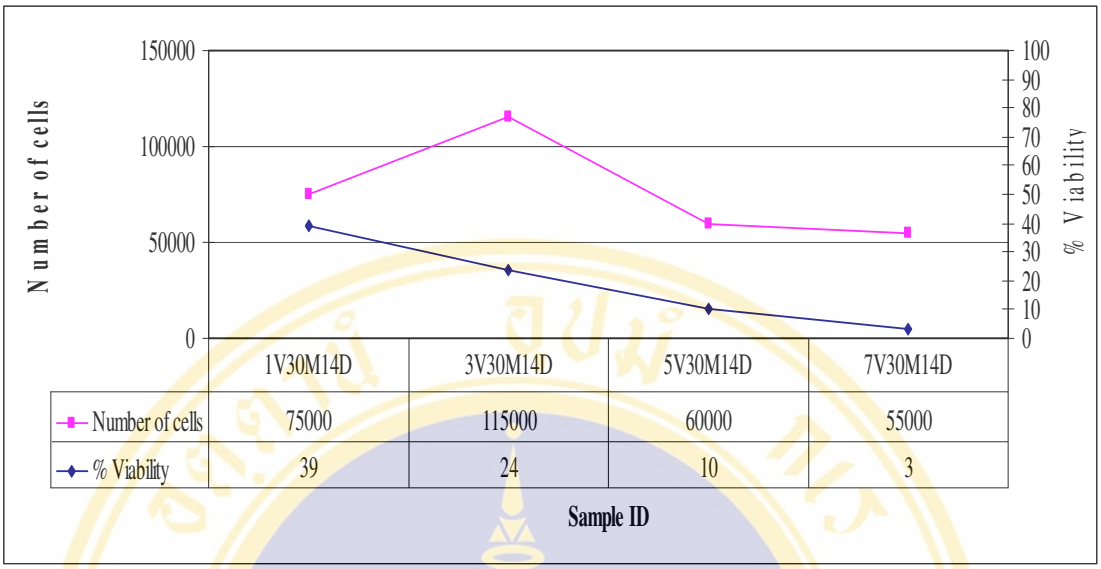
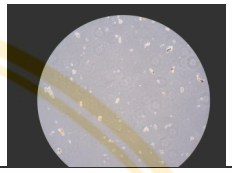
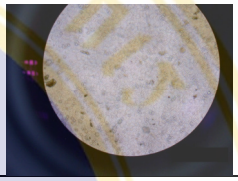
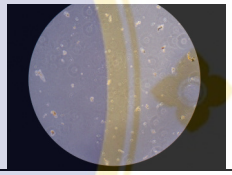
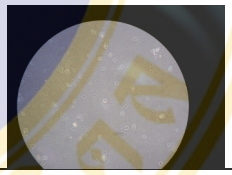


Figure 4.9 The number and % viability of cell repelled at various electrical potentials.

In order to estimate the proper repelling duration for all repellent, the fibroblasts on a substrate that culture for 14 days were subject to repel using a constant potential of -1 volts. The repelling time was varied from 15, 30, 45, and 60 minutes. The results were shown in table 4.7.

Table 4.7 The effect of repelling time to electrical cell repellent from conductive substrate by applying -1 volts of electrical potential at 14 days of culturing.

Sample ID	Repelling time	Result
1V15M14D	15 minutes	
1V30M14D	30 minutes	
1V45M14D	45 minutes	. 
1V60M14D	60 minutes	

The number of cell repelled from the conductive substrate at several repelling times and % viability of cells were counted using trypan blue assay. The results were shown in figure 4.10.

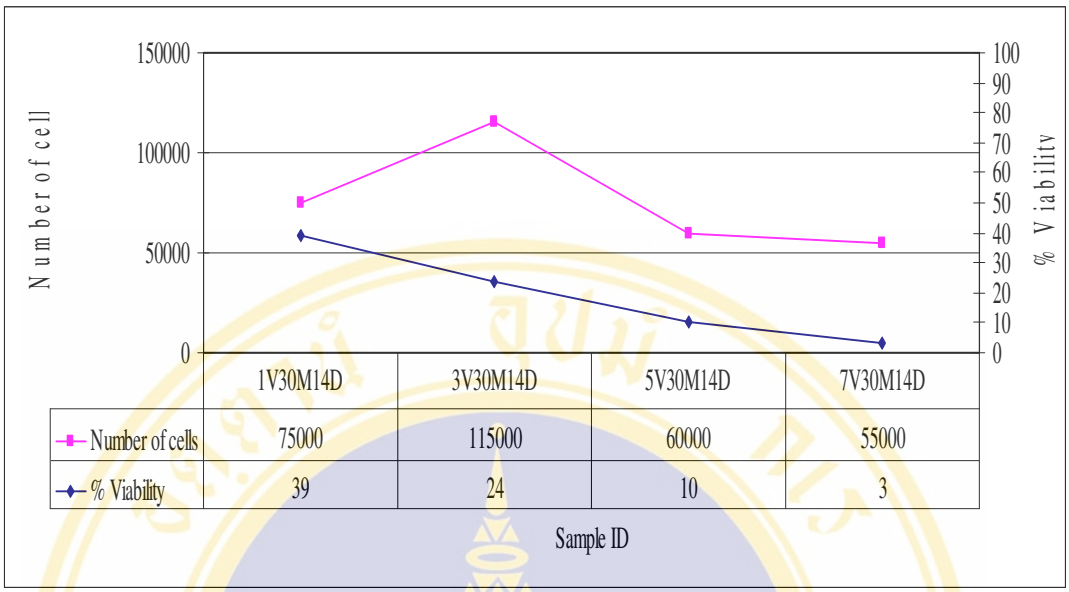
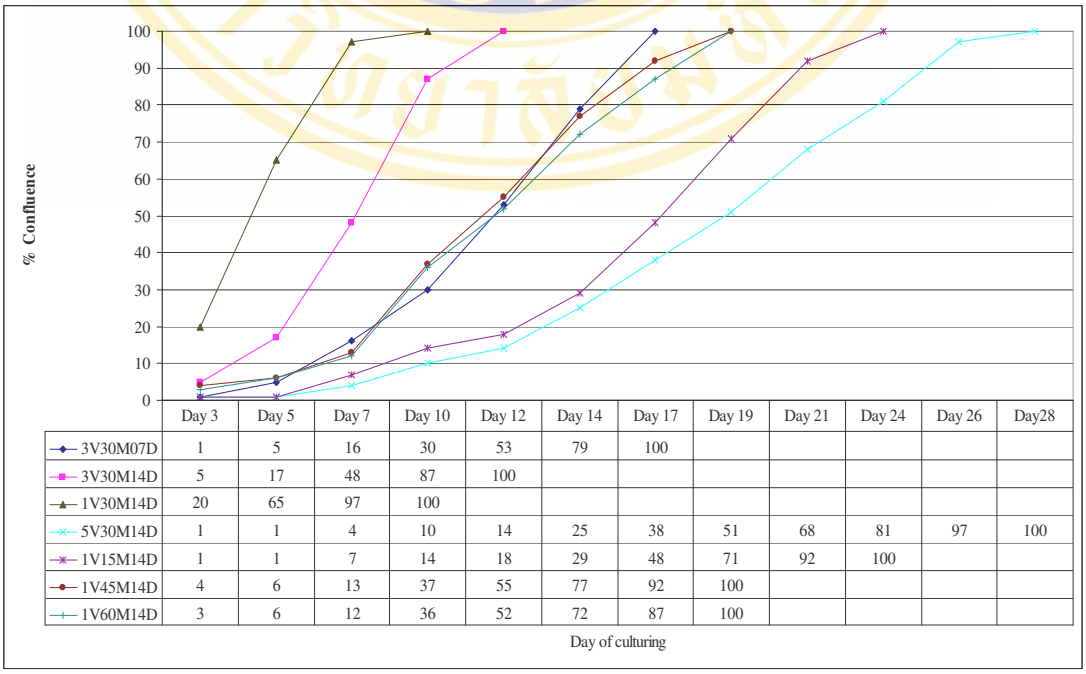


Figure 4.10 The number and % viability of cells repelled at variety of repelling times.

The repelled fibroblasts were then place to the new culture dish. The growth of cells was observed continuously by inverted light microscope in term of % confluence. The results were shown in figure 4.11.



	Day 3	Day 5	Day 7	Day 10	Day 12	Day 14	Day 17	Day 19	Day 21	Day 24	Day 26	Day28
3V30M07D	1	5	16	30	53	79	100					
3V30M14D	5	17	48	87	100							
1V30M14D	20	65	97	100								
5V30M14D	1	1	4	10	14	25	38	51	68	81	97	100
1V15M14D	1	1	7	14	18	29	48	71	92	100		
1V45M14D	4	6	13	37	55	77	92	100				
1V60M14D	3	6	12	36	52	72	87	100				

Figure 4.11 The % confluence of fibroblast repelled at various conditions.

CHAPTER V
DISCUSSION

5.1 The substrate fabrication

The conductive polymer substrate constructed from electrochemical deposition method shows the dark, brittle and thin structure (see figure 4.1). There are several factors affected the morphology of the polypyrrole substrates such as electrical potential and electrical current. These two parameters directly affected on the roughness and the thickness of the substrate [24]. Moreover, the size of polypyrrole is limited by the size of platinum electrode.

5.1.1 The effect of electrical potential on the substrate construction

The polypyrrole substrate constructed from the constant electrical potential rough surface. In the experiment, the substrates were constructed by applying the difference electrical potential, 0.7 volt, 0.8 volt, 0.9 volt and 1.0 volt respectively. These condition were chosen due to the reason that the electrical potentials below 0.7 volt gave the very slow substrate growing on the electrode while at the electrical potential above 1.0 volt showed the rapid polymerization and resulting in uneven films deposition on the electrode The sample fabricated using constant 0.7 volt shows the smoothest surface comparing with that of the sample fabricated using 0.8, 0.9 and 1.0 volt. The sample fabricated using 1.0 volt shows the highest roughness surface on SEM (see figure 4.2). The electrical potential applied to the electrodes cause the electrical field between the pair of electrodes and the charged particle of the conductive polymer moves along the direction of electrical field and deposits on the working electrode. A higher electrical potential used promotes a higher of electrical field and causing the fast and non-uniform deposition of substrate on the electrode. This results in the high roughness surface of the conductive polymer substrate. Moreover, the direction of electrical field is also effect on the formation of conductive substrate on the electrode. In the experiment, platinum plate was used as a working

electrode and platinum rod was employed as a counter electrode. The electrical field generated from these 2 electrodes is not uniform along the surface due to the distance from the counter electrode to the surface of working electrode is not constant. Resulting in the non-homogeneous of electrical field between the electrodes.Therefore, the deposition of conductive substrate is not uniformly and a high roughness of the substrate was obtained.

The surface morphology of the side that attaches to the platinum plate electrode showed a smoother surface than that of the side facing to the counter electrode at every electrical potential applied. This is because the polymerization of the pyrrole occur on the surface of the working electrode which means that the roughness of the substrate is the nearly the same as the roughness of the platinum plate electrode. Therefore, the roughness of polypyrrole surface attached on the working electrode are nearly the same for all sample fabricated using 0.8, 0.9, 1.0 volt.

In the experiment, the thickness of substrates fabricated at 0.7, 0.8, 0.9 and 1.0 volt for 1 hour were observed using scanning electron microscope. The thickness increases when the electrical potential increases (see table 4.1). This is because the thickness of the conductive substrate is definitely depends on the rate of polypyrrole polymerization and the rate of electrochemical deposition on the electrode. The substrate fabricated using a constant 0.7 volt shows the thinnest substrate which is approximately 50-60 nm whereas the sample fabricated using 1.0 volt shows the thickest polypyrrole layer (nearly 100-110 nm). Therefore, at the constant period of potential applied (1 hour), the 1.0 volt demonstrates the highest rate of electrochemical deposition and resulting in the highest thickness of the substrate. While the 0.7 volt of substrate fabrication shows the thinnest layer.

The thickness of conductive substrate is also proportional to the duration of electrical applied. The polypyrrole substrate fabricated at 0.9 volt for 1, 2, 3 and 4 hour(s) showed the thickness are 90-100, 100-110, 110-120 and 160-170 nm respectively (see table 4.2). While the electrochemical deposition proceeded, the substrate slowly and continuously polymerized on the electrode, resulting in the increasing of substrate thickness.

5.1.2 The effect of electrical current on the substrate construction

The study of the constant electrical current effects was carried out. The constant current of 10, 20 and 30 mA as well as the fabrication periods of 1, 2, 3 and 4 hour(s) were chosen for this study. The results of surface morphology and substrate thickness were observed using scanning electron microscope.

The surface morphology of The substrates prepared using the constant currents showed a smoother surface compare with the substrates constructed using a constant current electrical potential (as seen in figure 4.2 and 4.4). The rate of polymerization depends on the amount of charges presenting on the surface. Therefore, the substrates produced using constant current give a smoother surface compared to the substrate fabricated at constant potential where the electron density decrease as the thickness increased.

The surface morphology of substrate fabricated galvanostatically at 10, 20 and 30 mA for 1 hour showed the low roughness surface on SEM micrographs (figure 4.4). The surfaces of conductive substrate fabricated at various electrical currents are nearly the same due to the constant rate of electrochemical deposition. The surface roughness of the substrate is similar to the constant potential cause that is the backside of conductive substrate surface demonstrates the smoother surface than that of the front (see figure 4.5).

The thickness of substrate is also related to the electrical current applied. The higher the electrical current used, the thicker the substrate are. This is due to the large number of charge presence on the surface. The thickness of these substrate fabricated using 10, 20 and 30 mA of electrical current approximately are 5-10, 15-20 and 180-185 nm respectively.

By increasing the fabricated period at a constant 10 mA across the electrodes, the sample prepared for 1, 2, 3 and 4 hrs produced the conductive substrate with the thickness 5-10, 50-60, 60-70 and 150-160 μm respectively. These results show that the longer period of electrochemical deposition time yields a thickness the thicker layer substrate.

5.2 Cell culturing on the substrate

The biocompatibility of the conductive polypyrrole on various cell types has been studied elsewhere, including keratinocytes [41], neural cells [43-45] or endothelial cells [38]. The biocompatibility of polypyrrole on the fibroblasts was established by R.L. Williams [39]. This study reported that the polypyrrole does exhibit a good compatibility and allow a number of viability cells. However in this research, the fibroblasts were chosen for culturing and electrical repellent due to the availability and the cell growth characteristic. The $5x10^5$ fibroblast cells were load directly on the substrate fabricated using 30 mA electrical current for 30 minute due to the smoothness of the surface morphology on this sample as well as the high thickness of the substrate. After 5 days of culturing, The substrate with fibroblast was removed and placed to a new culture dish in order to avoid the error from cells attached on the culture dish. Since the non-transparency property of the conductive substrate, the growth of fibroblast on the substrate can not be observed directly. Thus, the image taken by the inverted light microscope as shown in the figure 4.6 only depicted the fibroblast that grow on the substrate and spread out to the perimeter of the substrate and on the culture dish latter on. Normally, fibroblast grows as a monolayer at the surface of supporting material and spread over the surface. According to the figure 4.6 (a) – (d), fibroblasts grow and adhere on the culturing plate after 2 days of culturing, the substrate was then placed to the new culturing dish after 5 days of culturing and continue growing until the optical micrograph shows that there is no fibroblast appearing on the substrate. Fibroblasts were grown and spreaded out to the edge of substrate as shown after 9 days of culturing. Finally, the fibroblast spread out on the culture plate after 14 days as seen in figure 4.6.

The other experiment which is used to study the growth of fibroblast on the conductive substrate is lactic acid test. Lactic acid is the waste product secreted from culturing cells. The lactic acid concentration in the culture media is direct proportional to the number of fibroblast growing on the substrate. According to the result of lactic acid test (figure 4.7), the lactic acid concentration of the sample containing fibroblast on polypyrrole substrate after 5 days of culturing was 3.52 mmol/L. Since the sample was removed from the culture plate and replaced on a new

culture dish, the lactic acid level reduced to about 1.15 mmol/L at day 7th. After 10 days of culturing, the lactic acid concentration of the sample is increased about 3.04 mmol/L due to the increasing of fibroblast. This lactic acid levels is almost constant until 14 days of culturing may due to the balance of cell death and cell growth on the polypyrrole substrate. The rate of lactic acid concentration increased rapidly again after 17 days of culturing due to the large number of fibroblasts populated over the polypyrrole substrate onto the culture dish. Furthermore, the lactic acid concentration is almost constant again between 19-24 days of culturing due to the balance of cell growth and cell death. After that, the death of fibroblast was observed through the lactic acid measurement. The over crowded cells growing on the substrate and the high accumulation of waste secreted from cells caused the lactic acid concentration drops down to 3.09 mmol/L after 26 days of culturing. However, the lactic acid concentration pattern of positive control differs from the test. At beginning, the lactic acid level of positive control is nearly the same as the test because of the same amount of fibroblast loaded to the surface. After that the lactic acid concentration raises up to the 5.94 and 9.98 mmol/L after 7 and 10 days of culturing respectively due to growth of cells. Then, the lactic acid concentration is constant until day 19th of culturing, the lactic acid concentration reduces to 5.51 mmol/L because of the death of cells. After that, the growing of fibroblast came to the stable state again. The lactic acid concentration of negative control, substrate and culture media, is less than 0.5 mmol/L on all measurement over the period of study. However, the lactic acid concentration measurement can not represent the growth of fibroblasts on the conductive substrate because the fibroblast spread out from the substrate to the surface of culture dish and grow on the culture dish surface so that the lactic acid concentration of the later culturing days include the lactic acid secreted from the fibroblast growing on both of the substrate and surface of culture dish.

5.3 Electrical cell repellent from the conductive substrate

The aid of electrical potential was employed across the conductive polypyrrole electrode and platinum plate counter electrode. Fibroblasts adhering on the conductive substrate were repelled and counted using trypan blue assay. The conditions of

repellent study were affected by the duration of repelling, the starting number of cells, and the electrical potential across the electrodes. A similar study was also study by Mian J. [56]. This research studied release of lipoplex, the combination between lipid and DNA, on the gold micro electrode surface by the aid of electrical potential. The results indicated that the mechanism of repellent is likely due to the electrostatic repulsion between lipoplexes and the negatively charged electrode surface.

5.3.1 Cell repellent at the variety of electrical potentials

The effect of electrical potential on the repellent was studied in this research. The fibroblasts on polypyrrole substrate cultured for 14 days were chosen for this study. The amount of electrical potential was varied for -1, to -3, -5 and -7 volt (s) and the repelling time was controlled as 30 minutes. The number of cells repelled from the conductive substrate at -1, -3, -5 and -7 volt(s) are $7.5x10^4$, $11.5x10^4$, $6.0x10^4$ and $5.5x10^4$ cells respectively and the % viability are 39, 24, 10 and 3 % respectively (see figure 4.8). According to the electrostatic repulsion, the intensity of the force field is directly depending on the applied voltage and charge particle. Moreover, this force field is inverse proportion to the dielectric constant of surrounding fluid and the distant between them [47]. That means the applied potential of -7 volts should give the strongest repulsion force field for fibroblast samples followed by -5, -3, and -1 volt respectively. However, the results indicated that at -7 volts of electrical repellent showed the lowest number of cell while the -3 volts is highest. These possibly due to the high potential applied across the sample, causing the generation of electrons move from cathode to anode. The movement of theses electrons from the cathodic electrode (conductive polypyrrole) to the anode penetrates the fibroblast on the polypyrrole substrate, resulting in the damage and lysis of fibroblast, thus, the % viability is reduced. Furthermore, at high electrical potential applied to the electrodes causes the fetal calf serum proteins in the culture media denatured which was observed from the bubble appearing around the electrodes. Moreover, these bubbles generated around the conductive electrode handles reduced the contacted surface area on the electrode, resulting in the lowest number of cell repelled fibroblast.

The percent viability of fibroblasts repelled at -1, -3, -5 and -7 volt(s) were 39, 24, 10 and 3 % respectively. Indicating that at lower potential applied, the number of

repelled cells and % viability are higher than that repelled using a higher potential. Another possibility that affected on the cell repulsion at various applied potential is the heat generation. According to the electrical power supplied from the electrodes, the higher electrical potential supplied to the media, the larger power is converted into heat. In case of this research, the heat generated between two electrodes, transfer to the culture media and resulting in the death of fibroblasts adhered on the conductive polymer electrode. The higher electrical potential was applied to the electrode, the more heat was generated and the more percent of cell death was.

5.3.2 Cell repellent on various culturing periods

Since the total number of cells cannot be directly observed on the conductive polypyrrole substrates due to their non-transparency property. The number of repelled cells and the percent viability of fibroblast are carried out at various intervals using trypan blue assay. The number of cell repelled from those day of culturing. The percent viability and the number of fibroblasts repelled after 7, 14 and 21 days of culturing using -1 volt for 30 minutes are $10.5x10^4$, $11.5x10^4$ and $9.5x10^4$ cells respectively. This result indicated that at day 14^{th} of culturing, fibroblast can grows on the conductive substrate better than 7 and 21 days of culturing. However, the lactic acid test on theses sample do not agree with the number of cell repelled from the substrate because the measured lactic acid concentrations from the samples are the lactic acid secreted from both fibroblast on the polypyrrole substrates and the fibroblast on the culture dish.

The percent viability of fibroblast repelled from the samples cultured for 7, 14, and 21 days with the electrical potential of -3 volts and 30 minutes are 23, 24 and 22 percent respectively (see figure 4.9). These percent viabilities are almost constant for a constant potential and repulsion period, indicating that the number of repelled cells may depends on the intensity of the electrical potential as well as the duration of repulsion periods. Thus the effects of these parameters are clarified in the next study.

5.3.3 Cell repellent at the variety of repelling times

By varying the repulsion duration from 15, 30, 45, and 60 minutes on the fibroblast samples cultured for 14 days with -1 volt of electrical potential was applied,

the number of repelled cells are $2x10^4$, $7.5x10^4$, $6.5x10^4$, and $7.0x10^4$ respectively (see figure 4.10). Typically, the longer repelling period, the larger numbers of repelled fibroblasts should be obtained. However, the results showed that the number of repelled cells was increased up to 30 minutes of repulsion, the number of repelled cells is then constant for the longer repulsion periods (30, 45, and 60 minutes). This possibly is the maximum number of cells that can be repelled at this condition.

The percent viability of fibroblast repelled at 15, 30, 45 and 60 minutes is 50, 39, 36 and 33 percent respectively (see figure 4.10). The effect of repelling time is inversely proportional to the percent viability of repelled fibroblast. Since a longer reeling time causes the accumulation of heat around electrodes resulting in the death of repelled fibroblast. Therefore, the longer repelling period applied to the electrodes, the lesser percent viability of repelled fibroblast is obtained.

After repellent, repelled fibroblasts at various conditions were load to the new culture dishes in order to observe the growth behavior of the cells. The percent confluence of fibroblast growing on culture dish was estimated under optical microscope. The results demonstrated that the high number of repelled cells provides the high percent confluence at the same period of culturing which explained by the large slope of confluence plots in figure 4.11. However, the percent confluence estimated from this study may not represent the growth characteristic of repelled cells after treat with the electrical field, since the number of repelled cells loaded on each culture dish is not the same.

CHAPTER VI
CONCLUSION

The conductive polypyrrole substrate fabrication using electrochemical polymerization method provided the dark, thin and brittle films. The surface morphology of polypyrrole doped with sodium dodecyl sulfate showed a higher roughness when synthesized using the constant electrical potential compared to the substrate synthesized using the constant electrical currents. The roughness of substrates increased when the applied electrical potential increases. Whereas the substrates surface morphology on the side that attached on the electrode showed a smoother surface than that facing to the counter electrode. Therefore, the roughness of substrate surface depends on the surface roughness of working electrodes. The thickness of polypyrrole substrates were evaluated on various factors i.e., fabrication time, the electrical potential, and electrical current. The thickness of the substrates constructed using various constant electrical potentials showed that the higher electrical potential used, the thicker the substrate was. Moreover, the long fabrication duration causing in the large accumulation of the polypyrrole on the substrates, therefore, the thickness is larger than that constructed at a shorter period. On the study of cell growth on these substrates, the results showed that the fibroblast can grow and proliferate on the conductive substrate without any problem. The appropriate electrical potentials for cells repelling in term of number of repelled cell and percent viability is -3 volts for 30 minutes. The 14 days of culturing provided the highest numbers of repelled fibroblasts.

Although, the effect of electrical stimulation can be employed in the cells repellent application but the results is still not satisfied because of the low number of cells repelled from the substrate comparing to the starting cells loaded on each substrate and the low percent viability of cell. Therefore, the future studies may need to be concentrated on the effects of applied potential such as the use of pulse electrical

potential instead of constant potential and the use of high voltage generators in order to increase the electrostatic force with low heat generation that may damage the cells.

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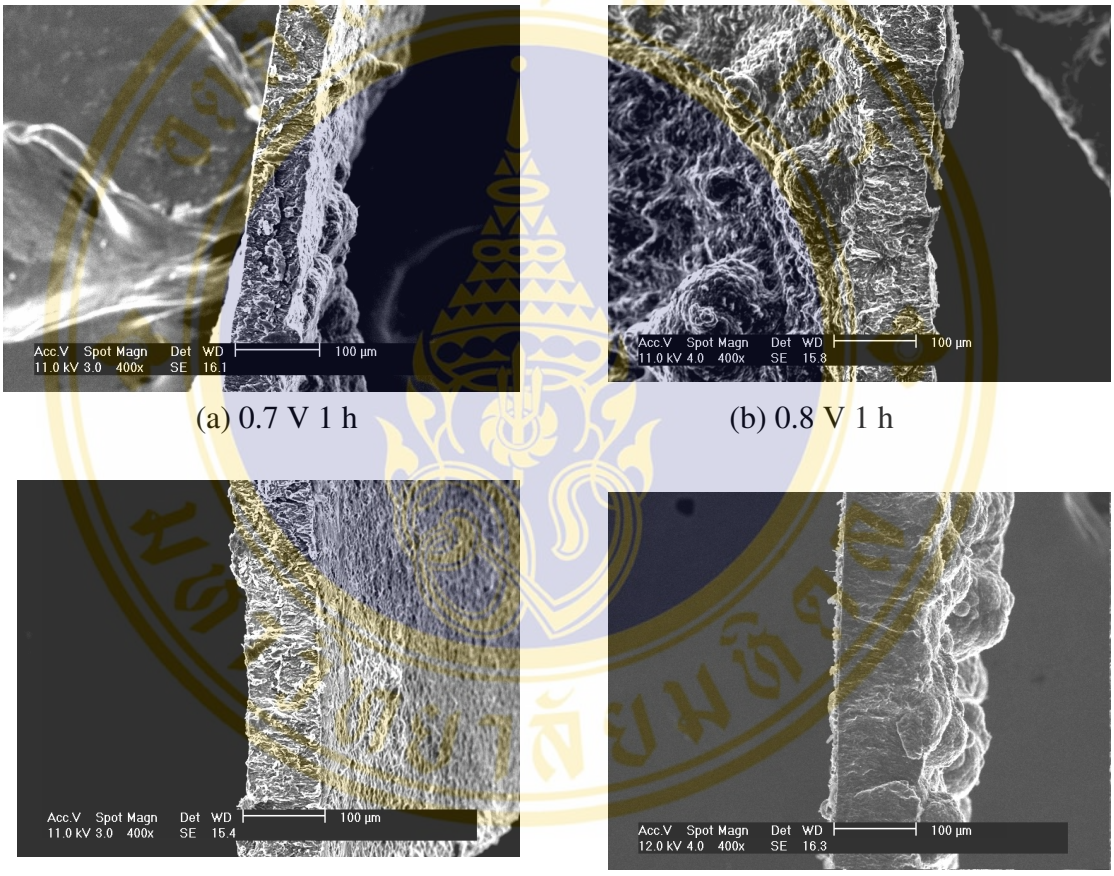
APPENDIX

APPENDIX

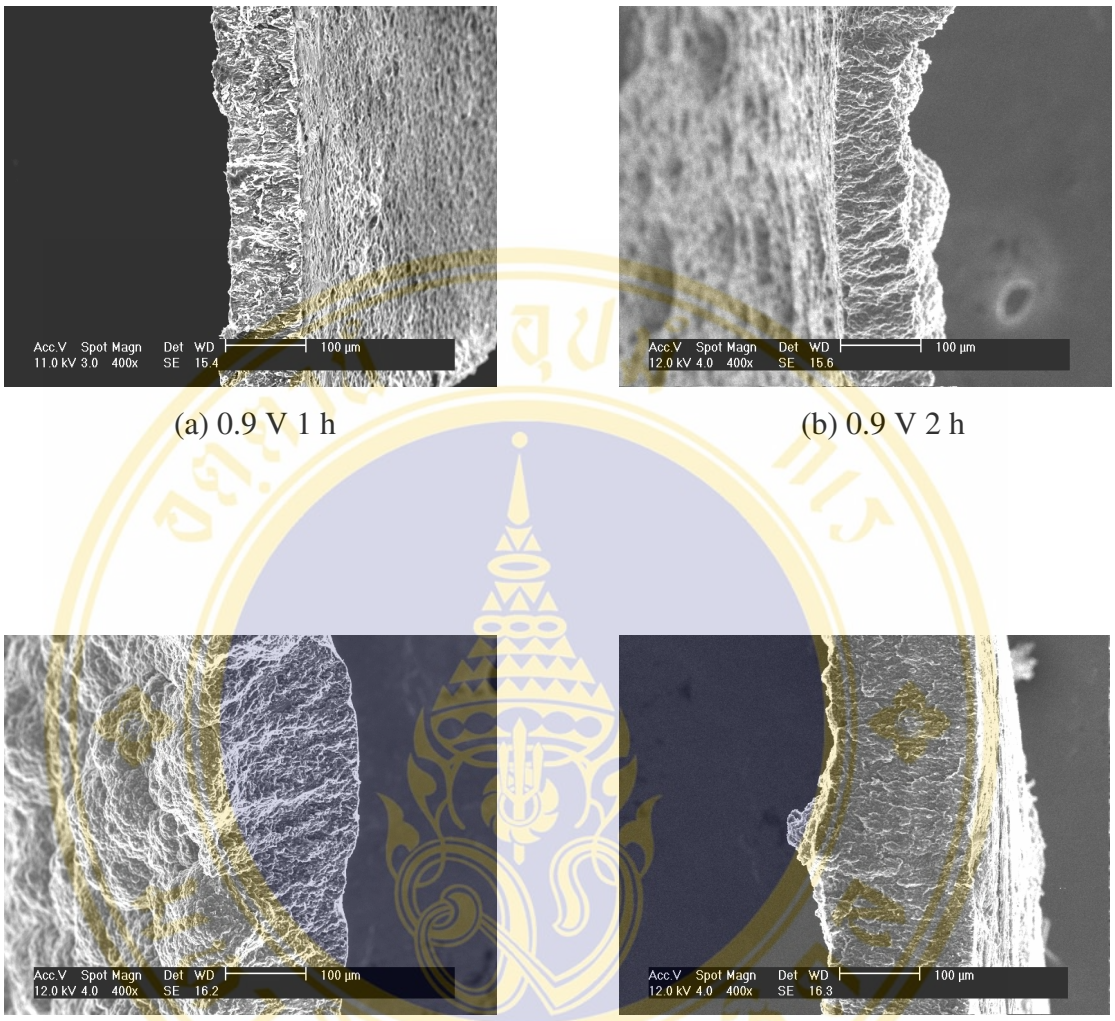
Cross section SEM micrographs of polypyrrole



(a) 0.7 V 1 h (b) 0.8 V 1 h

(c) 0.9 V 1 h (d) 1.0 V 1 h

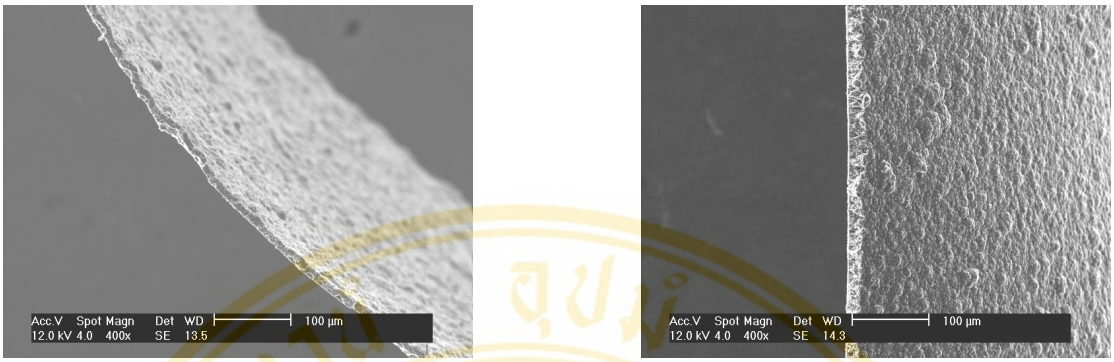
Figure 1 Thickness of polypyrrole substrates constructed from various amount of potentials.



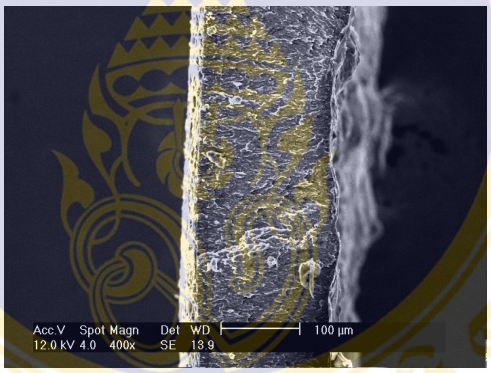
(a) 0.9 V 1 h (b) 0.9 V 2 h

(c) 0.9 V 3 h (d) 0.9 V 4 h

Figure 2 Thickness of polypyrrole substrates constructed from various period of times.



(a) 10 mA 1 hr (b) 20 mA 1 hr



(c) 30 mA 1 hr

Figure 3 Thickness of polypyrrole substrates constructed from various amount of currents.

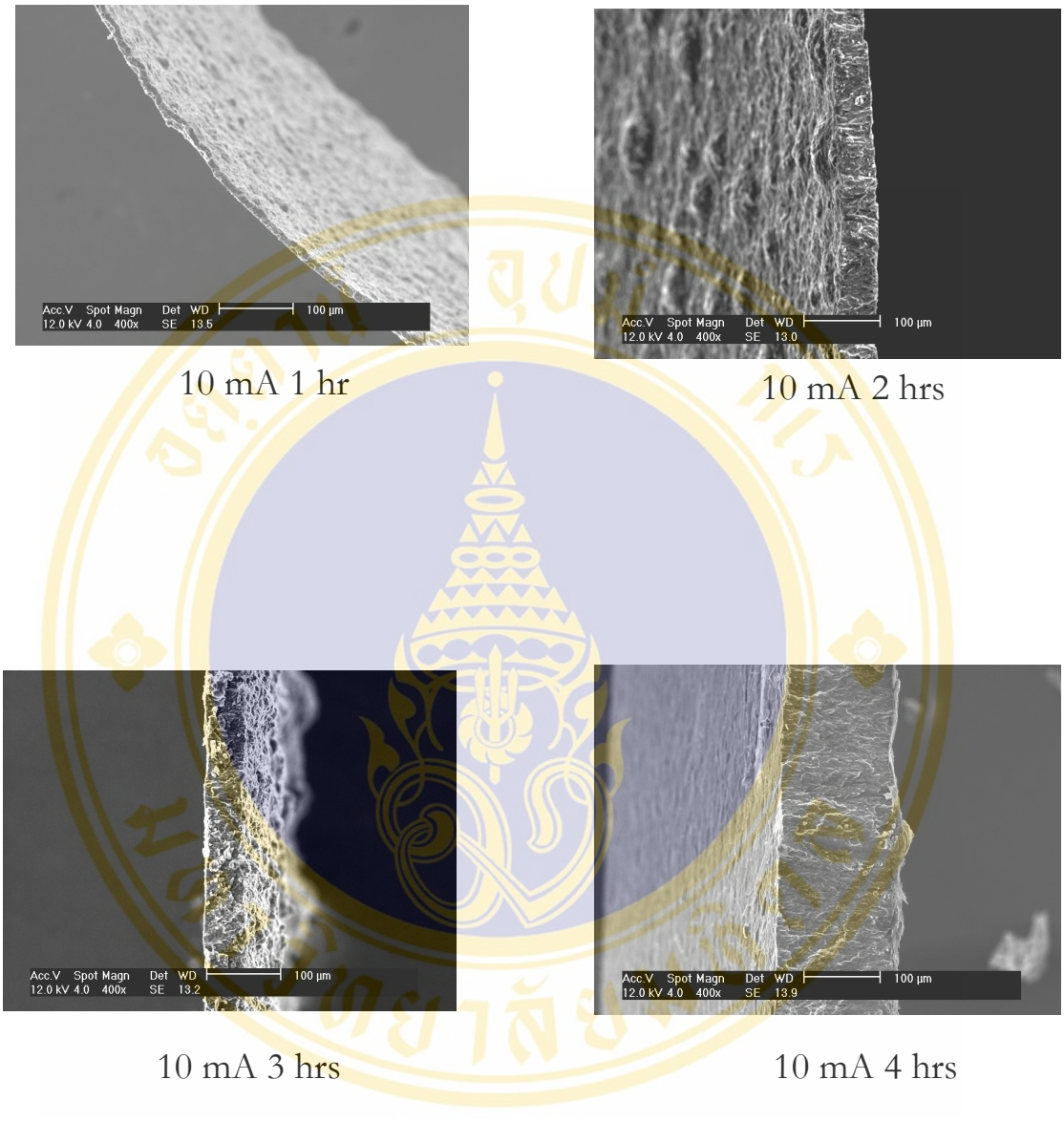


Figure 4 Thickness of polypyrrole substrates constructed from various period of times.

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