MODULATION ÉLECTRIQUE DES FONCTIONS CELLULAIRES PAR LE BIAIS D'UN NOUVEAU BIOCONDUCTEUR DÉGRADABLE

ELECTRICAL MODULATION OF CELLULAR FUNCTIONS USING A NOVEL BIODEGRADABLE BIOCONDUCTOR SUBSTRATE

Thèse présentée à la Faculté des études supérieures de l'Université Laval dans le cadre du programme de doctorat en médecine expérimentale pour l'obtention du grade de Philosophiae Doctor (Ph. D.)

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Résumé

Le but de cette étude est de produire un biopolymère conducteur utilisable en génie tissulaire. Pour y parvenir, nous avons préparé un biomatériau conducteur à base d’acide polylactique (PLA) et de polypyrrole (PPy). Les analyses physicochimiques montrent que le biopolymère est bien structuré et malléable sans être fragile. Le PPy est distribué sur toute la surface de la membrane d’acide PLA assurant une conductivité continue. Lorsqu’un champ électrique est appliqué au biopolymère, les fibroblastes prolifèrent beaucoup plus comparativement au biopolymère sans champ électrique. Les analyses ultrastructurales confirment l’adhésion et un phénotype normal (élongation cellulaire, formation de dendrites, petit noyau, petit cytoplasme) de fibroblastes prolifératifs. Suite à une stimulation électrique, des médiateurs inflammatoires (IL-6 et IL-8) sont modulés sur le plan génotique et protéique. Conclusion: L’ensemble de ces travaux démontre que l’application d’un champ électrique à la surface d’un biomatériau biocompatible et conducteur favorise l’adhésion, la prolifération et la structuration des fibroblastes humains. Ces travaux suggèrent le potentiel conducteur des biopolymères en génie tissulaire.
Abstract

Electrical stimulation (ES) has a long history as an alternative clinical treatment and as an effective technique to modify cellular behavior. However, a functional biomaterial must be used when applying ES to tissue engineering, i.e., to localize an electrical field within a well-defined 3-D space. This material must be conductive, biocompatible, electrically stable, and biodegradable. This thesis focuses on the design and preparation of a novel degradable bioconductor and the demonstration of its mediatory effect on the efficacy of ES in regulating cell functions. The thesis consists of five chapters. Chapter 1 is the general introduction, which provides the background information, rationale, hypotheses, and objectives. Chapter 2 describes the materials and methods for the entire experiments, including materials design, preparation, physical-chemical characterizations, cytocompatibility tests and electrically stimulated cell cultures. Chapter 3 presents all the experimental results. Chapter 4 is the discussion, and chapter 5 is the conclusions and perspectives. As a result, a degradable bioconductor made of PPy/PLA was successfully prepared. This bioconductor displayed adjustable conductivity, acceptable electrical stability, and cytocompatibility. A multi-well electrical cell culture plate was designed and fabricated to host electrically stimulated cell cultures and to monitor electrical parameters. A weak DC electrical field mediated by the bioconductor provides an effective technique to regulate cellular adhesion, viability, proliferation, gene expression, and protein secretion. This degradable bioconductor mediated ES highlights potentially significant biotechnological applications in tissue regeneration and others.
Foreword

This thesis includes five chapters as follows: general introduction, materials and methods, results, discussion and a general conclusion. Regarding the thesis contents, I designed and performed the experiments, as well as wrote the manuscripts. Most of them are published in the following journals:

   
   Dr. Rouabhia, Mahmoud: discussed the experiments and revised the manuscript;
   Dr. Wang, Zhaoxu: discussed the experiments;
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<tr>
<td>ES</td>
<td>Electrical stimulation</td>
</tr>
<tr>
<td>EF</td>
<td>Electrical field</td>
</tr>
<tr>
<td>DC</td>
<td>Direct current</td>
</tr>
<tr>
<td>ECPs</td>
<td>Electrically conductive polymers</td>
</tr>
<tr>
<td>PPy</td>
<td>Polypyrrole</td>
</tr>
<tr>
<td>PLA</td>
<td>Polylactide</td>
</tr>
<tr>
<td>PDLLA</td>
<td>Poly(d,l-lactide)</td>
</tr>
<tr>
<td>PLLA</td>
<td>Poly(l-lactide)</td>
</tr>
<tr>
<td>PD</td>
<td>Petri dish</td>
</tr>
<tr>
<td>ITO</td>
<td>Indium-tin oxide</td>
</tr>
<tr>
<td>TE</td>
<td>Tissue engineering</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>XPS</td>
<td>X-ray photoelectron spectroscopy</td>
</tr>
<tr>
<td>Rs</td>
<td>Surface electrical resistivity</td>
</tr>
<tr>
<td>DBS</td>
<td>Dodecylbenzenesulfonic acid sodium</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>IL-8</td>
<td>Interleukin-8</td>
</tr>
<tr>
<td>GADPH</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcription</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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CHAPTER I

INTRODUCTION
1.1. Introduction

Regulating cellular activity is of the utmost importance when regenerating cells and tissues with normal biological structures and functions. For example, the healing of skin largely relies on the timely migration and proliferation of fibroblasts and keratinocytes, with the latter settling on the outermost layer of the epidermis. This process is regulated by various kinds of biological factors such as pro-inflammatory cytokines (1-3). To date, several techniques have been developed to modulate cell functions using mostly biological cues, including signaling proteins such as cytokines and growth factors, or chemicals such as nitrogen oxide (4-7).

As a physical cue, electrical stimulation (ES) may constitute a potentially efficient approach to regulate cellular functions. This assumption is based on three facts. First, bioelectricity is present in the human body and plays an important role in maintaining normal biological functions. Second, numerous cells in the human body are sensitive and are intricately tied to electrical behaviour, including, most obviously, nervous system cells and cardiac cells. Finally, ES has been used in the clinical setting as means to promote wound healing (8, 9), reduce chronic pain (10), and treat diseases such as Parkinson’s (11, 12). However, an effective method using ES for the purpose of tissue regeneration has yet to be conceived.

Tissue engineering (TE) is a promising revolutionary approach in regenerating damaged tissues or organs by constructing a living cell scaffold substitute. It thus occurred to us that a combination of ES with TE might provide a novel technique to direct cell activity within the scaffold in vitro and to enhance tissue regeneration in vivo. However, to achieve such a goal, i.e., to electrically stimulate cells that are immobilized within a scaffold, a new biomaterial must be designed. This material must be electrically conductive, biocompatible, easily processable, electrically stable, and ideally, biodegradable. This review therefore focuses on ES and cell activity, tissue engineering, conductive polymers, and biodegradable polymers, in which the rationale of this project is discussed.
1.2. Electrical stimulation and cellular activity

1.2.1. Endogenous bioelectricity and life

Electrical phenomena widely exist in living organisms and play an essential role in maintaining normal biological functions. One obvious example is the electric eel, a fish found in South America that is able to generate an electric shock as high as 500 V and 1 ampere, which is strong enough to use when hunting prey, for self-defense purposes, and even to stun a man. In the human body, most phenomena are closely related to electrical activity, such as, for example, thinking, the beating of the heart, and muscle contraction. Endogenous electrical field (EF) also plays an important role in wound healing. When the skin is cut, a steady electrical current and transepithelial potential difference (TEP) is immediately set off between the skin lesion and the inner tissues, lasting from hours to weeks depending on the healing process (13). It is believed that TEP can enhance wound healing and nerve regeneration by directing and stimulating cell physiological functions, such as epithelium migration, proliferation, secretion, and nerve outgrowth (14).

Bioelectricity is also vital to life development. Life itself actually begins with a change in cell membrane potential. When a zygote is formed by the merging of a sperm cell and an egg cell, ion channels in the egg membrane are activated, thereby preventing the access of other sperm cells (15).

What is bioelectricity? Bioelectricity refers to the electric potential or current produced by or occurring within cells, tissues, and living organisms (14). As the basic unit for maintaining biological functions, the cell is also, from an electrical perspective, a “microdynamotor” (15). Even at rest, a living cell can maintain its electrical potential (called resting potential, approximately -70 mV for neurons) due to the difference in ion concentration (Na\(^+\) and K\(^+\)) between the inside (negative charges) and outside (positive charges) of the cell membrane (15). Like a battery, the maintaining and changing of electrical potential in the cell membrane involves the movement of ions, rather than electrons, across the membrane. The cell membrane is semipermeable and can selectively control ion diffusion. When stimulated, cells
create an action potential by changing the permeability of their membrane to ions. In the case of nerve cells, electrical signals generated by the sensory organs travel along the nerve fibres of sensory neurons to the brain for processing, and the signals containing operating information are fed back through motor neurons to the targeted cells or tissues in response.

In medicine, monitoring the electrical activity of the brain or the heart is a routine practice. For example, the electrocardiogram (ECG) is used to check if the heart is normal. The waves of electrical activity produced by the heart are monitored and analyzed by fixing electrodes to specific sites on the skin. Doctors can therefore determine the presence of cardiac arrhythmia, acute myocardial infarction, or hyperkalemia. Similarly, the electrical behaviours observed when scanning the brain (a procedure called electroencephalography or EEG), provide valuable information for use in psychology and psychiatry as well as in diagnosing brain tumours.

1.2.2. Electrical stimulation and cell activity

1.2.2.1. Basic research

Research on the effects of ES on cell activity involves a wide range of parameters, including the type of cells, the cellular behaviour to be measured, and probably most importantly, the manner in which ES is applied. Obviously, not all cell types and cellular behaviours display the same sensitivity to a particular type of ES. At present, only a few ES devices have been designed to investigate the effects of ES on cell morphology (16), migration (17), orientation (18, 19), division (20), proliferation (16), gene expression (21, 22), protein synthesis, and secretion (23, 24). The following review examines the types of devices.
A. Salt-bridge ES system

This device is used extensively in the electrophysiological assay (Figure 1.1) (20, 25, 26). McCaig et al. opted for this device (14, 20, 27). Typically, cells are cultured within a narrow channel formed by two parallel coverglasses placed in a cell culture Petri dish. Two agar-gelled salt bridges connect the culture medium to saline solution contained in two beakers, followed by a connection to a direct-current (DC) power source through two electrodes (e.g., Ag/AgCl electrodes) (14).

**Figure 1.1.** Schematic illustration of a salt-bridge electrical device. 
*A*: top view of chamber. *B*: side view of the ES device (14).
Zhao et al. (28) applied a DC EF of 150 mV/mm to stimulate bovine corneal epithelial cells (CECs) and found that these cells oriented perpendicularly to the EF vector and migrated toward the cathode at an average rate of approximately 15 μm/h (17). Interestingly, in a serum-free medium, the cells showed no orientation under ES. The authors also reported that the cell division of the CECs was oriented and that the cell cleavage orientation was perpendicular to the EF vector (20).

Stewart et al. (27) used this device to study neurite growth from embryonic Xenopus laevis at various EF intensities (0-200 mV/mm). They reported EF-enhanced neurite growth and the turning and branching of the neurites toward the cathode. The neurite turning was dependent on EF intensity. A range of EF at 143-200 mV/mm significantly increased the proportion of cathode-turning neuritis, compared to that observed with EF at 120-133 mV/mm. The authors studied the coeffects of ES and various calcium channel inhibitors on neurite growth and found that the EF-induced and -oriented nerve growth was modulated by calcium channel subtypes and intracellular calcium concentration. McCaig et al. (25) studied the coeffect of EF (intensity range 0-200 mV/mm) and of various neurotrophins on neuron growth in Xenopus laevis embryos. The EF-induced cone growth was enhanced by several neurotrophins. The nerve growth facing the cathode was more rapid and the directed branching was promoted under the ES/neurotrophin coaction. Using the same device, Erskine et al. (26) applied a DC EF of 50-133 mV/mm to study the collaborative effects of endogenous neurotransmitters and EF on neurite growth in Xenopus embryos, and reported that EF induced faster neurite growth than did the control, and that neurites facing the cathode grew faster than did those facing the anode. The application of d-tubocurarine, a nicotinic AChR (acetylcholine receptor) antagonist, inhibited the field-induced cathodal orientation of the cultured neurites, whereas atropine, a muscarinic AChR blocker, and suramin, a P2-purinoceptor antagonist, markedly enhanced the guidance properties of the applied field. The author also demonstrated that the EF-induced neurite orientation was supported by the activation of growth cone nicotinic AChRs by self-released acetylcholine.
**B. Three-band ES system**

Qiu *et al.* (29) constructed another device to electrically stimulate cell cultures (Figure 1.2), in which two thin indium-tin oxide (ITO) membranes patterning on glass were used as electrodes. Three bands, ITO-Gap-ITO, were allowed to culture cells (rat bone marrow stromal cells). This device is used primarily to study cell responses to electrical charges built up on the electrode surface. The authors reported that the positively charged ITO surface (anode) enhanced cell attachment under 0.8 V at 24 h, suppressed their ability to spread, and significantly reduced the osteopontin expression and ALPase activity.

**Figure 1.2.** Modified illustration of the “three band” (ITO-Gap-ITO) ES system. (A) Top view of the setup; (B) Cross section of the culture chambers (29).
**C. Three-electrode electrochemical reaction system**

This device is a variation of the typical three-electrode electrochemical cell reaction system, as shown in Figure 1.3 (21). It contains three electrodes: a working electrode made of conducting membrane, such as ITO (16, 21, 24) or a conducting polymer (30, 31) polymerized on either an ITO-coated Petri dish or glass, a platinum or gold counter-electrode, and a reference electrode such as Ag/AgCl. Cells are cultured on the surface of the working electrode and are electrically stimulated.

**Figure 1.3.** Schematic illustration of a 3-electrode electrochemical reaction device. Potential is applied to the working electrode with a potentiostat through a function generator that generates rectangular pulse waves (30).
Yaoita et al. (16) applied an ITO electrode to stimulate HeLa cells and found that a potential higher than +0.7 V modulated cell viability, membrane permeability, and cytoskeleton, while the cells showed no response to a potential of less than +0.5 V. At +0.6 V, the cells were round in shape, yet remained viable. An interesting result was reported by Kimura et al. (21) that a rectangular wave stimulation upregulated the neurite outgrowth of PC12 cultured on an ITO surface in the absence of NGF. The authors suggested that EF played a role partially similar to that of NGF. Further study of the mechanism revealed that the effects of ES on neurite outgrowth may be related to the PKC cascade (21). Langer’s group (30, 31) at MIT used a conducting polymer, such as polypyrrole, as the working electrode to stimulate cells. Wong et al. (30) applied -0.5 V to stimulate bovine aortic endothelial cells cultured on fibronectin-PPy, showing that the cells attached but maintained their round shape, compared to the spindle shape culture observed on the control (without ES). The authors concluded that ES had changed the surface property of the PPy, which in turn modulated cell functions. Schmidt et al. (31) reported that PC12 cells cultured on PPy under ES for 2 h showed a significant increase in neurite length compared to that of the controls (PPy without ES). It was therefore proposed that this conducting polymer could be used in nerve regeneration.

Other ES devices have been investigated, such as those using simply two metal electrodes (32) and a capacitively coupled mode (33). Aside from steady EF, pulsed EF of various intensities and frequencies has also been studied (22, 34).

1.2.2.2. Clinical applications

In the clinical setting, ES has been used as an alternative source to enhance wound healing (9, 35), including treating arterial diabetic chronic ulcers (36), reducing chronic pain caused by arthritis and headache (10), and treating diseases such as Parkinson’s (11, 12) and other neurological disorders (37), to name but a few. For example, Lagrange et al. (38) studied the effect of ES in the treatment of Parkinson’s disease. Sixty patients with an average of 14 years of Parkinson history participated in the study. The quality of life related to the disease was assessed by the Unified
Parkinson’s Disease Rating Scale I (UPDRS I) method. The author applied bilateral subthalamic nucleus deep brain stimulation (STN-DBS) at a pulse width of 60 μs and a frequency of 130 Hz. The voltage at several volts was increased progressively over the first few postoperative days. Twelve months after surgery, the UPDRS I scores showed that ES significantly improved all aspects of quality of life, including the motor (+48%), systemic (+34%), emotional (+29%), and social (+63%) dimensions.

1.2.3. The effects of ES on cellular activity

Many groups have studied the effects of ES on cellular activity. However, the true mechanism is still not well understood, thus remaining the subject of debate. For example, regarding cell adhesion and migration, some studies suggested that cell responses to ES were due to the static attraction between charges on the electrode and on the surface of the cell membranes (29), whereas Zhao _et al._ argued that they were associated with protein participation (17, 28). These authors found no cell migration or orientation in serum-free medium, thereby concluding that the interaction between the ES and the proteins in serum enhanced cell migration and orientation. However, Qiu _et al._ (29) reported that in serum-free medium, more cells attached onto the anode than onto the cathode, indicating that the static attraction between the positive charges on the anode and the negative charges on the cell membranes played a role. Another example involves the EF effect on nerve regeneration. Patel and Poo (19) reported that neurites of single dissociated Xenopus neurons grew faster toward a cathode under a DC EF of between 0.1 and 10 V/cm, while Cork _et al._ (39) observed an anode-oriented growth of PC12 neurites. In contrast, Davenport _et al._ (40) demonstrated some growth cones (completely formed dendrites) oriented cathodally, whereas some showed no response. McCaig (14) concluded that sensory neurites do not turn, motor neurites turn cathodally, and PC12 neurites turn anodally. Schmidt (31), however, observed no orientation and migration of PC12 neurites under ES. Therefore, understanding the orientation of neurites in an EF warrants further investigation.
It is certainly difficult to attempt comparisons between studies because of the different types of ES devices (21, 29) and various ES protocols, such as DC (31) versus AC (34), or different EF intensities and time frames. Different devices may signify different ES effects and mechanisms, resulting in a variety of applications. This should be taken into consideration when different results are compared. For example, McCaig’s device (14) applied two salt bridges both as electrodes and to connect power source with cell culture medium. When connected to a power source, ions or charged biomolecules in the medium will move to electrodes of opposite charges. Finally, a balance is formed in the medium, resulting in a non-uniform distribution of ion concentrations. The various ingredients in the culture medium may also affect cell activity differently, namely in terms of migration and orientation. For Qiu’s device (29), it is more than likely that the cells responded primarily to the electrical charges on the surface of two parallel membrane electrodes. In a study by Aizawa (21), cells were cultured on the surface of an ITO electrode membrane, and a Pt electrode was placed in cell culture medium. The electrical potential and/or vertical electrical current through the cells therefore caused the primary effect on cell functions. When the applied potential is high enough, electrochemical reactions may occur on the electrodes, and the resulting oxidation-reduction products may in turn affect cellular activity. Cell type (which varies in the literature) is another factor that complicates cross-publication comparison. Among others, excitable cells, such as neurons, may be more responsive (14) than non-excitatable cells. Even using the same type of cells, different cell passages and cell phases could produce a different response to ES (14), which may explain why McCaig observed cellular orientation (14), while Schmidt did not (31).

Therefore, to fully investigate cellular responses to ES, two questions must be addressed: How will EF be applied (device, DC/AC, intensity, duration, frequency, etc.), and is there any change in culture medium which may affect normal culturing (movement of ions and proteins, redox products, or changes in culture medium, such as temperature and pH)?
To study the cellular response mechanism to ES, several experiments have explored the role of ion channels by using ion-channel blockers. For example, Stewart and McCaig et al. (27) examined the coactivation of ES and different calcium and calcium channel inhibitors on neurite growth, and found that the EF-induced and -oriented nerve growth was modulated by calcium channel subtypes and intracellular calcium concentration. Erskine and McCaig (26) studied the synergetic effects of endogenous neurotransmitters and EF on neurite growth in Xenopus embryos. The authors reported that the application of a nicotinic AChR antagonist inhibited EF-induced neurite orientation, and that the application of a muscarinic AChR blocker enhanced the EF-induced effect. They indicated that the EF-induced neurite orientation may be mediated by the activation of growth cone nicotinic AChRs by self-released acetylcholine. Koyama et al. (24) cultured astroglial cells on an ITO-coated working electrode and found that the NGF production of astroglial cells was promoted by ES. The authors suggested that the ES-induced NGF release was perhaps associated with the Ca\(^{2+}\) dependent PKC pathway, as the application of both LaCl\(_3\) (a non-specific Ca\(^{2+}\) channel inhibitor) and phorbol 12-myristate 13-acetate (PMA) (a down-regulating agent of phorbolester PKC activity) suppressed NGF production, compared to the ES effect. Moreover, no significant difference was observed between the decreased NGF production using staurosporine (an inhibitor of PKC and other protein kinases) and that using PMA. Unfortunately, no direct evidence showed that the ES-induced NGF secretion was mediated by the PKC pathway via the Ca\(^{2+}\) channel. In addition, the total NGF release was the net outcome of a competition between the inhibitor-induced suppressive effect and the ES-induced augmentation. Furthermore, the suppressive effect largely relied on the inhibitor concentration. Unfortunately, the correlation between inhibitor concentration and NGF reduction was not reported.

In summary, research indicates that ES has the capacity to regulate cellular functions, promote wound healing, and enhance tissue regeneration; these factors therefore render ES highly attractive for tissue engineering applications.
1.3. Tissue engineering

1.3.1. Introduction

Four main approaches currently exist in clinical therapy to repair damaged or diseased tissues that require grafting: autografts from patients, allografts from other human donors, xenografts from other species, and artificial prostheses (41). Each of these strategies has its own limitations, such as insufficient donor sites (autografts) or donors (allografts), immune responses (allografts and xenografts) and the risk of transmission of inter-species diseases (xenografts), or the incapability to be fully integrated into the host tissue (synthetic grafts). Under such circumstances, tissue engineering (TE) proposes a potentially revolutionary technique to address these limitations (42). TE shows potential in regenerating rather than transplanting specific tissues or organs. Research activities in TE have increased tremendously in recent years. The market potential of TE is estimated to be more than $350 billion annually worldwide (43).

1.3.2. Brief description

The term "tissue engineering" (TE) originated in 1987 at the bioengineering panel meeting held by the National Science Foundation (NSF) (44), where TE was identified as an emerging technology and a highly promising field. In 1988, its definition was further defined at a special NSF-sponsored TE meeting (45). Today, a commonly accepted definition of TE is that expressed in 1993 by Langer and Vacanti as "an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ" (42).

TE is a multidisciplinary approach, involving biology, materials, engineering, and medicine. The general goal of TE is to regenerate diseased tissues or organs to restore their biological functions. A general approach in TE is to construct a biological substitute combining living cells within an artificial 3-D scaffold made ideally of
biodegradable polymers, with the goal of maintaining, repairing, replacing, or enhancing part of or the entire function of impaired tissues or organs (i.e., skin, bone, cartilage, nerve, and kidney). Following the implantation of the living substitute, the scaffold biodegrades and is gradually absorbed, relinquishing more space for further cell proliferation. Finally, the damaged tissue is restored or replaced by the newly generated tissue, which displays a structure and biological functions similar to those of the original tissue. In short, this approach mainly consists of two components: cells and scaffold. Various strategies exist to promote the cell/scaffold interaction, including the use of bioreactors, growth factors, and gene modification.

Another strategy in TE is the microencapsulation of cells within a polymer microsphere or macrocapsule matrices. Typically, cells are embedded in a polymer gel made of, for example, alginate (46, 47), chitosan (48, 49), or PEG (50), to form a cell suspension, which is then extruded into a coagulant solution or non-solvent to form semipermeable microspheres with cells inside. These microspheres can be implanted as vehicles to deliver therapeutic products secreted by the cells. The semipermeable polymer membrane has an appropriate pore structure that allows the exchange of nutrients and waste through the membrane by diffusion, while protecting the cells from being attacked by immune cells. The cell encapsulation strategy has shown potential in the treatment of diabetes (51), cancer (52), and disorders of the central nervous system (53).

In addition to therapeutic applications, TE shows potential in the area of diagnostics, as tissue constructed through in vitro culture can be used as a biosensor in the screening of novel drug candidates, or for testing drug metabolism and uptake (53, 55).
1.3.3. Main research areas

1.3.3.1. Cells

Cells are key components in TE. The regeneration of any tissue relies on its cellular functions, including cell adhesion, migration, differentiation, proliferation, and secretion of biomolecules such as extra-cellular matrix. Cell-to-cell signalling and cell-substrate interaction also play an important role.

Cell sourcing is critical in TE. Cells can be sourced from autologous, allogeneic, and xenogenic tissue. Autologous cells are most often preferable because of their biocompatibility to the host. However, in cases where a large amount of cells is required for initial seeding, sufficient autologous cells are not always available. Under such circumstances, rapid cell expansion becomes vital. Moreover, it is essential for the primary cells to maintain their normal biological functions without altering the phenotype following multiple cell divisions in long-term culture (56).

Stem cells are a particularly significant cell source in TE because of their superior ability to differentiate into different types of cells to regenerate virtually all types of tissues or organs. Human stem cells are primal cells (57, 58), and they can maintain the ability to proliferate for long-term divisions without altering cell phenotype, and can potentially transform into a wide range of desirable cell types (59, 60), such as fibroblasts, neurons, and osteocytes. Stem cell research consists of stem cell isolation, derivation, and specific cellular differentiation by growth factors, or of other physical and chemical approaches. Fully understanding the factors that direct stem cell differentiation will generate critical knowledge and bring about innovative techniques to further develop TE.

1.3.3.2. Scaffold materials and biodegradable polymers

The artificial scaffold acts as a temporary cell growth substitute for the natural extracellular matrix (ECM). During tissue regeneration, the scaffold degrades simultaneously with cell proliferation and the deposition/reorganization of ECM, with
the degradation products being absorbed or eliminated. Finally, the newly generated functional tissue replaces the scaffold. Scaffolds therefore play an important role in TE by supporting cell attachment, guiding cell migration and tissue growth, providing a 3-D space for new cells and tissue, and mediating other cues such as mechanical stress. Furthermore, scaffolds can be used as drug delivery systems for the controlled release of growth factors or other functional biomolecules to modulate cellular activity.

It is therefore highly advantageous that scaffolding biomaterials display the following properties: (1) acceptable biocompatibility, i.e., not evoking intolerable tissue response; (2) adequate biodegradability, with a controllable degradation rate to match cell/tissue growth; (3) bioresorbable degradation products; (4) suitable surface properties that enable cell attachment, proliferation, and secretion; (5) a 3-D interconnected network with suitable porous structures to direct cell growth and the exchange of nutrients and metabolic waste; (6) appropriate mechanical properties; and (7) suitable processability.

A wide spectrum of biomaterials have been investigated, ranging from inorganic materials, such as ceramics (61, 62), natural polymers, including collagen (63, 64) and chitosan (65, 66), microorganism-produced materials like poly(hydroxybutyrate) (PHB) (67), and synthetic biodegradable polymers such as polyanhydrides, polyorthoesters and polyamides. Today, the most widely studied synthetic biodegradable materials are polyesters, such as poly(glycolic acid) (PGA) (68-70), poly(lactic acid) (PLA) (71-73), and copolymer PLGA (74-76), thanks to their established biocompatibility, absorbable degradation products, and adjustable degradation rate. These biodegradable polymers have been widely investigated as scaffolding materials in various applications involving skin (77, 78), bone (79, 80), cartilage (81, 82), and nerves (83, 84). Moreover, several multi-functional biomaterials are now available, including materials capable of releasing biomolecules to regulate cell differentiation and proliferation (85), polymers containing a specific peptide sequence (86, 87), such as RGD (88), used to enhance cell recognition and
attachment, as well as those with specific architectures to facilitate the formation of vasculature. Synthetic polymers have also been combined with naturally occurring materials to form hybrid biomaterials (76).

1.3.3.3. Cell/scaffold graft

The basic premise of TE is to form a biologically viable graft made of cells grown on a scaffolding substrate, followed by a cell-to-tissue transformation that occurs either in vitro in a bioreactor or in vivo following implantation. Used to mimic the in vivo environment, the bioreactor is a cell culture system capable of culturing an entire tissue or organ through its sophisticated control of cell/scaffold interactions and medium exchange. In order to determine the architecture and biological functions of newly generated tissue, certain standards must therefore be established, such as identifying the specific features, efficacy level, and safety norms required for engineered tissue, as well as the packaging/shipping standards regarding bioartificial substitutes.

1.3.3.4. Bioactive molecules

To regulate such cellular functions as adhesion and differentiation within a scaffold, a wide range of bioactive molecules, such as growth factors (89-91), cytokines (92, 93), and other peptides (94, 95) have been combined with scaffolds to constitute controlled drug release systems. For example, Woo et al. developed a drug delivery system by combining human bone morphogenetic protein-2 (BMP-2) with PLGA microspheres to study the drug release effects on local bone formation in the rabbit, and found that the sustained delivery of BMP-2 from the PLGA microsphere system resulted in a more rapid and better healing of the bone. Murphy et al. combined vascular endothelial growth factor (VEGF) with a porous PLGA scaffold and reported that the sustained release of VEGF from the scaffold induced the proliferation of endothelial cells in vitro (96, 97).
1.3.4. Clinical applications and challenges

Although TE offers a promising way to create new tissues, achieving this goal remains a challenge. One major obstacle is the vascularization (98, 99) within the newly generated tissue, particularly highly metabolic tissue requiring significant blood supply, such as cardiac muscles and liver and kidney tissue. A variety of strategies have been developed to promote the formation of vasculature in scaffolds by applying various angiogenic growth factors (100) released from the biodegradable scaffolds, combinations of various vascularization cells (101), and novel polymer designs with vascular-like architectures (102). In addition to biological factors, mechanical stimulation has also been used (103). It was reported that vascular smooth muscle cells in a vascular graft under pulsatile perfusion were capable of synthesizing more collagen, resulting in blood vessels with greater mechanical strength than that observed under standard static culture conditions (104, 105).

Cell sourcing is essential in TE. The following issues must therefore be addressed: harvesting of sufficient primary cells, cell expansion without altering the phenotype and normal biological functions, and multiple cellular layer formation. The remodeling of cells and ECM associated with the biodegradation of the scaffold and the implantion in host site must also be taken into consideration. Moreover, the immune acceptance of the artificial tissue cannot be overlooked. Nevertheless, advances in stem cell research are expected to have a significant impact on TE.

Despite these challenges, exciting advances have been made in TE, ranging from skin substitutes, bone and cartilage grafting to metabolic tissue such as liver, heart, kidney, and pancreas, to the regeneration of peripheral nerves using nerve guidance channels. Various engineered tissues are currently in preclinical trials (such as ChondroMimetic, by OrthoMimetics Ltd., Cambridge, UK), while others are in the regulatory evaluation process (106). Among the most successful TE products are skin substitutes (107, 108) to treat ulcers and burns, including TranCyte, Dermagraft, and Apligraf. For example, TransCyte, manufactured by Advanced Tissue Sciences Inc. in La Jolla, California, is the first FDA-approved engineered tissue product, entering
the market in 1997. This skin substitute integrates human newborn fibroblasts on a biodegradable polymer, and has been applied as a temporary wound dressing for the treatment of burns. Organogenesis Inc., located in Massachusetts, has produced Apiligrاف, a bi-layered human skin equivalent composed of living epidermal layers and dermal cells, which are integrated with collagen and used to heal ulcers.

1.3.5. Summary

TE represents an approach to potentially revolutionize many medical practices by constructing living cell/scaffold substitutes. A key challenge in TE lies in how to successfully regulate cellular activities using an appropriate scaffold and/or bioactive molecules. The application of ES through a conductive scaffold may provide an alternative method of regulating cellular behaviour. In order to mediate ES through a scaffold, we must design a functional material that is both electrically conductive and biodegradable.

1.4. Conductive polymers and polypyrrole

1.4.1. Introduction

Organic polymers are usually insulators. For example, electrical wires are wrapped by a layer of plastics to prevent them from short-circuiting. However, with the discovery of a new category of inherently electrically conductive polymers, this notion is no longer valid. Although still under development, these novel polymers are similar to the plastics of the 1950s in that they show enormous potential for applications in various areas.
1.4.2. Electrically conductive polymers (ECPs)

1.4.2.1. Brief history

In 1958, Natta et al. were the first to synthesize polyacetylene (PA). The final product was a black powder with relatively low conductivity ($10^{-11}$ to $10^{-3}$ S/m) (109). In 1977, Shirakawa et al. (110) developed a silvery thin film of PA with a conductance $10^3$ times higher than that of Natta’s model. Further studies showed that when PA was weakly oxidized by iodine, it turned a golden colour with a dramatically higher conductivity of over $10^9$ folds that of the original one. In addition, other electrically conductive polymers with heterocycles, such as PPy (111), have been developed, which are more stable in ambient environment and more easily processable than their PA counterpart. Research is now focusing on how to improve the electrical stability and processibility of the EPCs and to explore their industrial applications. The 2000 Nobel Prize in Chemistry was awarded to Professors Alan J. Heeger, Alan G. MacDiarmid, and Hideki Shirakawa for their significant contribution to the discovery and development of conductive polymers (112).

1.4.2.2. Properties of ECPs

ECPs are organic polymeric semiconductors with wide-ranging conductivity from approximately $10^{-10}$ to $10^{-7}$ S/m (inorganic polymer semiconductors, such as poly(sulphur nitride) showing high conductivity at extremely low temperatures ($T_c = 0.26K$), are not included in this category). ECPs have generated a great deal of interest in the last 20 years because they simultaneously demonstrate the physical and chemical properties of organic polymers and the electrical characteristics of metals. The extensively studied ECPs (shown in Figure 1.4) are polyacetylene (PA), polypyrrole (PPy), polyaniline (PANI), polythiophene (PT), poly(p-phenylene) (PPP), poly(para-phenylene vinylene) (PPV), and their derivatives. A common feature of ECPs is the alternation of single and double bonds (conjugation) along their molecular backbone. The counterions incorporated in the oxidized polymer chain, called dopants, play a critical role in the high conductivity of ECPs; for example, doped PA has a conductance $10^9$ times higher than that of non-doped PA.
1.4.2.3. Charge transfer mechanism (112, 113)

Mobile charge carriers are required for a material to display conductivity. In metal, the mobile units are electrons and a high density of free electrons can move easily from atom to atom under an EF. Moreover, metallic atoms are arranged in a regular pattern and comprise a highly ordered, crystalline 3-D network structure. Metal-atom d orbitals can overlap with the equivalent orbitals of neighbouring atoms in all directions, which enables the electrons to become highly mobile. For example, the conductivity of copper is approximately $10^8$ S/m.

In ECPs, the charge carriers are solitons, polarons, or bipolarons rather than electrons, which are generated by a doping process. The conjugated system makes for an entire delocalization of the p orbital electrons along its molecular chain, resulting in a continuous path of overlapping p orbitals. The movement of charge carriers along the conjugated polymer chain under an EF and the hopping of charges between adjacent chains are responsible for the conductivity of ECPs.

![Common conductive polymers](image)

**Figure 1.4.** Common conductive polymers.
The electrical conductivity of ECPs, \( \sigma \) (S/cm), is given as a function of charge carrier density and carrier mobility, as follows:

\[
\sigma = e (n^- \mu^- + n^+ \mu^+)
\]

where \( n^- \) and \( n^+ \) are electron and hole density (cm\(^3\)), \( \mu^- \) and \( \mu^+ \) are electron and hole mobility (cm\(^2\)/Vsec), and \( e \) is the electric charge quantity of a carrier (1.602 \times 10^{-19} \text{ Coulomb}), respectively (112).

### 1.4.2.4. ECPs in nature (114-116)

Electrically conductive polymers (ECPs) have also been found in various organisms. For example, melanins (pigments), a biopolymer found in plants, fungi, and animals, are composed of polyacetylene, polypyrrole, polyaniline, and/or their copolymers. In humans, they are present in the skin, eyes, hair, and inner ear, and play an important role in determining human skin colour and protecting cells from being damaged by solar UV radiation, high temperatures, heavy metals, or oxidizing agents. They also mediate the immune responses and conduct light, heat, and electricity.

### 1.4.2.5. Applications

Because of their electrical conductivity and redox activity, ECPs have been investigated for use in numerous applications, including antistatic materials (117), paper, photography, fabrics, electromagnetic shielding (118), diode transistors, capacitors (119), light-emitting diodes (120, 121), rechargeable batteries (122), and gas sensors (123), as well as in biomedical applications as biosensors (124), microactuators (125), and controlled release systems (126).
1.4.3. Polypyrrole (PPy)

1.4.3.1. Introduction

Of all known conducting polymers, PPy is the most extensively studied because of its easy synthesis and long-term ambient stability, despite the fact that its conductivity does not match that of PA. Although studied longer, PA has been found to be easily oxidized by oxygen in air and is unstable in a humid environment, therefore limiting its applications. Moreover, PPy can be synthesized in aqueous solution and doped simultaneously with relatively high conductivity. Furthermore, recent studies have shown that PPy has demonstrated acceptable biocompatibility both in vitro (127), and in vivo (128), making it very attractive for biomedical applications.

1.4.3.2. Brief history

In 1916, PPy was chemically polymerized for the first time with the oxidant H₂O₂ (129) as an amorphous black powder called pyrrole blacks. Very little interest was generated thereafter regarding this powder because of its non-structural property and poor conductivity. In 1963, Weiss et al. (130) reported an iodine-doped PPy with a significant conductivity of close to 100 S/m. Continuous PPy films were then prepared electrochemically by Dall’Ollio in 1968 (131), and later, in the 1980s, electrochemical polymerization was further developed to obtain PPy with a high conductivity through doping. Since then, research on PPy has focused on improving its electrical conductivity, mechanical properties, and stability (132-134). In recent years, PPy has also been extensively investigated in biomedical areas, particularly in biosensors (124).

1.4.3.3. Chemical structure

Pyrrole, the monomer of PPy, is a five-membered heterocyclic ring with the chemical formula C₄H₅N, as shown in Figure 1.5. It has an aromatic structure with delocalized π-electrons throughout the ring. Pyrrole is also a basic subunit of many important biological molecules, such as heme, bile pigments, and certain amino acids and alkaloids. PPy is a chemical compound connected by a number of pyrrole rings
through 2,5-positions. PPy also exists naturally, particularly as an ingredient of melanin.

![Chemical structure of pyrrole.](image)

**Figure 1.5.** Chemical structure of pyrrole.

### 1.4.3.4. Synthesis of PPy

PPy is synthesized primarily by electrochemical or chemical polymerization. Pyrrole monomers can be oxidized into PPy by either an oxidant or oxidative electrical potential, as shown in Figure 1.6, where A stands for anion, and n the polymerization degree, which is usually unknown. Pyrrole is very easily oxidized and many oxidants, such as acid, peroxide, and oxidative transition metal ions are used. The most common oxidant for PPy polymerization is ferric salts, such as FeCl₃, Fe(CIO₃)₃, and FeBr₃ (135, 136). Oxidized PPy is a polycation, combing the counterions (such as Cl⁻) from the dopant. In the electrochemical method, PPy is polymerized directly on the surface of the electrode.

Electrochemical polymerization, currently the most widely used method of preparing PPy for biomedical studies, offers many advantages: it can easily produce PPy with a high conductivity and can also coat it *in situ* on rather complex geometries. The electrochemical method does, however, have its limitations. It is difficult to prepare a large amount of PPy at one time, as polymerization is carried out on the surface of a
rather small electrode. Furthermore, electrochemically synthesized PPy possesses very little if any processability. In contrast, the advantages of chemical polymerization include the flexibility and the processability of the prepared PPy on a large scale and at a low cost. The chemical method also makes it feasible to combine PPy with other flexible polymers, thereby providing an efficient way to improve the mechanical properties of PPy.

![Figure 1.6. Polymerization of PPy.](image)

### 1.4.3.5. Charge carriers (112, 113)

PPy obtains its high conductivity through a doping process, in which charge carriers are generated. Typically, there are two types of doping for ECPs, namely, p-doping and n-doping. P-doping is formed by an electron removed from the valence band, which generates a positive charge or hole, whereas n-doping is created by inserting an electron into the conduction band, resulting in a negative charge. N-doping is less common because it is very easy to be oxidized by oxygen in air.

Depending on the oxidation degree, doped PPy has two types of charge carriers called polarons and bipolarons, which are the basic conducting units (Figure 1.7). When PPy is oxidized, an electron is removed from the nitrogen of a pyrole ring, generating a radical cation (hole) and a chain deformation, usually across the neighbouring 3-4 pyrrole rings. This radical cation (called polaron) then attracts a counterion to achieve charge neutrality. It is delocalized because of the Coulomb attraction or a local
change in the equilibrium geometry of the bond deformation. A bipolaron is formed when PPy is further oxidized and the unpaired electron in a polaron is removed. The bipolaron can also be generated by the union of two independent polarons between neighbouring chains through a charge transfer reaction. Polarons or bipolarons travel along a conjugated chain and pass the defect or jump to the neighbouring chains by thermally activated hopping or by a tunneling effect under EF. As counterions are not mobile, the conduction of PPy requires a high density of counterions. This is also why relatively high doping is required to achieve significant conductivity.

1.4.3.6. Ion-exchange and switching properties

PPy has two reversible states, namely, oxidation and reduction states (Figure 1.8). A switch between these two states is accompanied by ion exchange with the environment. In its oxidized state, the conjugated PPy molecular backbone is positively charged and incorporates anions (dopants) to achieve charge neutrality. PPy is reduced when its molecular chains release the dopant anions or incorporate cations to balance the anions, followed by a degeneration of conductivity. There are
two possible ways for ion exchange to occur: When PPy is doped with a large immobile anion $A^-$, such as dodecylbenzene sulfonate (DBS), and in an electrolyte solution containing small cations $C^+$, cations are inserted or removed at reduction or oxidation, respectively (Figure 1.8A). When PPy is doped by small mobile anions and in an electrolyte containing both small anions and cations, anions are removed at reduction or inserted at oxidation (Figure 1.8B). In the first case, the insertion of cations at reduction increases the volume of PPy, whereas in the second, the removal of anions at reduction decreases its volume (137).

![Figure 1.8. Reversible conversions between the reduction and oxidation of PPy.](image)

1.4.3.7. Physico-chemical properties

A. Conductivity

The conductivity of PPy is wide-ranging, from non-conducting state to $10^5$ S/m, depending on its doping level and preparation methods, such as chemical or electrochemical polymerization. Dopants, including type and amount, have the most significant contribution to PPy conductivity. This conductivity is also affected by its conjugation degree, which is altered by chain length, chain defects, crystalline
degree, interchain contact, and charge transfer interactions. In addition, environmental factors, such as temperature and moisture, influence its conductivity.

**B. Mechanical property**

Pure PPy is rigid, non-fusible, and insoluble in any solvent. It is usually obtained in the form of powder through chemical oxidation, or as thin films through electrochemical polymerization. To improve its processability, PPy is often combined with other materials: for example, PPy deposited on electrodes through electrochemical polymerization as sensors (138), or chemically coated on the surface of fabrics, resulting in a conductive and flexible membrane (132).

**C. Electrical degeneration**

The two main mechanisms affecting PPy electrical stability are extrinsic stability (133, 139) and thermodynamic stability (134, 140). The instability of the first mechanism is caused by exposing PPy to external ambient agents, such as oxygen, water, and peroxides, which attack the charged bonds of PPy, causing the deprotonation of pyrrole ring and the deterioration of conductivity. PPy conductivity can also degenerate over time, even in a dry, oxygen-free environment. This is caused by thermodynamic degradation, in which the doping counterions are gradually lost and the conjugation is degenerated, resulting in reduced conductivity.

**1.4.3.8. PPy in biomedical applications**

**A. Microactuators**

Microactuators are based on the reversible volume change of PPy, which is caused by ion insertion or deinsertion during electrochemical oxidation and reduction (137). The volume change can be as large as 10%. The simplest microactuator is a bilayer device, consisting of a conductive PPy layer deposited on a flexible substrate such as gold. When PPy is oxidized or reduced, the volume change of the PPy layer forces the substrate to deform (bend or stretch), changing electrical energy into mechanical force. The direction and magnitude of the mechanical force can be manipulated
through the appropriate PPy-substrate configurations. Examples include microtweezers (125) and microrobots to sort small particles such as cells (141).

**B. Biosensors**

In PPy-based biosensors, bioactive molecules are immobilized in PPy films through various mechanisms, such as doping, covalent binding, or physical entrapment, while at the same time retaining their biological functions. Aside from acting as an immobilizing substrate to prevent biomolecules from leaching out, PPy also measures or transfers the physicochemical signals generated during the reactions between substrate and the immobilized biomolecules. These signals can be either electrons or ions generated in a redox reaction, or the change in resistivity or optical property of PPy caused by analysts. The following types of biosensors have been extensively studied: catalytic sensors based on immobilized enzymes (124, 142, 143), immunosensors based on immobilized proteins (antigen-antibody reaction) (144), and DNA sensors based on immobilized single-strand DNA oligomers (145, 146). Besides their specificity and sensitivity, which are comparable to that of other conventional biosensors, PPy biosensors are relatively inexpensive, fast to react and easy to prepare, and are accurately controlled by electrical potential and current. One example of an effective application is the glucose-oxidase enzyme/PPy biosensor. PPy is deposited on the electrodes by a pulsed potential technique in electrolytes containing the enzyme that is entrapped in the PPy film during polymerization. The glucose-oxidase oxidizes glucose and generates hydrogen peroxide, which then oxidizes iodide ions to form triiodide ions. This reaction results in a change in PPy conductivity, which is proportional to the peroxide concentration. The glucose concentration in the solution can be calculated by detecting the change of PPy conductivity on the electrode. Another example of a highly promising application is immunosensing, in which antibodies (or antigens) are immobilized in PPy film. The antibody-antigen reaction alters PPy’s capacity or resistance, which is then electrically detected. As an alternative to standard ELISA, immunosensing is cheaper and faster, and could provide a label-free, real-time, colour-free measurement in clinical diagnostics.
C. Controlled drug release system

When charged drugs are incorporated in PPy as dopants or counterions, these drugs can be precisely released from the PPy through an electro-controlled process (126, 147-149). Wadhwa et al. (126) studied an electro-controlled drug release system, in which PPy was integrated with the ionic form of the anti-inflammatory drug dexamethasone (Dex), and was then deposited on electrodes by electrochemical polymerization. The drug was subsequently released in PBS using cyclic voltammetry (CV). The results showed that the release of the drug was controllable by the amount of CV cycles, and that the released drug reduced the amount of reactive astrocytes as effectively as did the controls. Xu et al. (148) developed a novel controlled drug delivery system by applying PPy/Au bilayer actuators as valves on a set of drug reservoirs. The drugs were pre-stored in the reservoirs and were released by electrically opening the bilayer flaps.

D. Tissue engineering

Regulating cellular activity in scaffolds is vital to tissue regeneration, in order to increase cell proliferation, accelerate ECM deposition, and recruit other types of cells by secreting various types of cytokines. Today, this regulation is most often achieved through cell-substrate interactions, for example, by mimicking natural ECM and by incorporating growth factors to support and regulate cellular functions. However, a variety of excitable cells and tissues in the human body are intrinsically responsive to electrical signals, such as neurons and smooth muscle cells. Other types of cells, such as fibroblasts and bone cells, are also electrically responsive, as reviewed in previous sections. Therefore, considering its electrical conductivity, unique redox properties, and biocompatibility both in vitro and in vivo, PPy is definitely attractive for use in biomedical applications. Furthermore, ES through PPy has already demonstrated the potential to enhance cell growth (31). PPy therefore detains an enviable position as a material platform to apply electricity as a physical cue to promote tissue regeneration and healing.
On the other hand, the high degree of conjugation in the molecular backbone of PPy makes it very rigid, insoluble, and poorly processable. It is almost impossible to use PPy alone as a structural material. Despite this, however, most recent studies on PPy in biomedical areas continue to use pure PPy films prepared by electrochemical polymerization. One efficient way to improve mechanical property and processability is to combine PPy with other type of polymers. Recently, a wide range of conductive PPy composites were developed from non-biodegradable conventional polymers such as poly(methyl methacrylate) (150, 151), poly(ethylene terephthalate) (132), and polyvinyl alcohol (152), particularly for industrial applications. Therefore, in keeping with these advances in the field, our group has developed a new concept of degradable bioconductor for biomedical applications.

1.5. Biodegradable polymers

1.5.1. Introduction

Research on biodegradable polymers dates back to the early 1950s, with the synthesis of poly(glycolic acid) (PGA) from glycolic acid. At that time, material degradation was negatively viewed because of the deterioration of mechanical properties and performance over time. These materials consequently found little use, compared to other synthetic polymers such as polyethylene. However, this instability over time turned out to be extremely important years later in certain biomedical applications. In the 1960s, the first synthetic absorbable suture made of biodegradable PGA came into market: the Dexon (by the Davis & Geck subsidiary of the American Cyanamid Corporation). Since that time, a series of biodegradable polymers have been developed with various explored applications, including their use in controlled drug release systems and, more recently, as scaffolding materials in TE.

Biodegradable polymers are either organic or inorganic, natural or synthetic, bacterial-synthesized or chemically synthesized. Chemically synthesized organic biodegradable polymers are among the most extensively studied because of their
tunable mechanical property, surface chemistry, and degradation rate, and because of their lot-to-lot reproducibility and non-immunogenicity. Common biodegradable polymers include polyesters, polyanhydrides, polyorthoesters, and polyamides. Among these, the most studied are a class of aliphatic polyesters, which have structures similar to those of bacterial-produced polymers, such as PHB, and which consist primarily of polylactide (PLA), polyglycolide (PGA), poly(ε-caprolactone) (PCL), and their copolymers. This class of biodegradable polymers has well-recognized biocompatibility. Their biodegradation products (e.g., lactic acid) exist in the human body and can be eliminated through its normal metabolism. As the first class of synthetic biodegradable materials, their clinical use as sutures was approved by the FDA in the 1970s. These aliphatic polyesters are the most extensively studied and commercialized synthetic biodegradable polymers, with an impressive number of medical applications (153-156).

1.5.2. Primary synthetic biodegradable polymers

1.5.2.1. Polyglycolide (PGA)

Polyglycolide (PGA), also called poly(glycolic acid), is a biodegradable thermoplastic polymer and the simplest linear aliphatic polyester (Table 1.1). It can be prepared beginning with glycolic acid by polycondensation or from glycolide by ring-opening polymerization. The latter yields materials of high molecular weight. As shown in Table 1.2 (157), PGA exhibits a high degree of crystallinity (approximately 45-55%) and is not soluble in most organic solvents. PGA has a glass-transition temperature of 35-40°C and a high melting point of 220-230°C. Known as the first synthetic absorbable suture material, it was marketed as Dexon in the 1960s. PGA fibres display high strength and modulus. Following implantation, these fibres lose their strength in 4 weeks and can be completely absorbed within 4 to 6 months. Due to its stiffness, PGA is commonly combined with polylactide, forming PLGA copolymers for biomedical applications.
Table 1.1. Structures of important biodegradable polymers.

<table>
<thead>
<tr>
<th>Polymers</th>
<th>Abbr.</th>
<th>Monomer structure</th>
<th>Polymer structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyglycolide</td>
<td>PGA</td>
<td><img src="image" alt="PGA monomer structure" /></td>
<td><img src="image" alt="PGA polymer structure" /></td>
</tr>
<tr>
<td>Polylactide</td>
<td>PLA</td>
<td><img src="image" alt="PLA monomer structure" /></td>
<td><img src="image" alt="PLA polymer structure" /></td>
</tr>
<tr>
<td>Polylactide-glycolide</td>
<td>PLGA</td>
<td><img src="image" alt="PLGA monomer structure" /></td>
<td><img src="image" alt="PLGA polymer structure" /></td>
</tr>
<tr>
<td>Polycaprolactone</td>
<td>PCL</td>
<td><img src="image" alt="PCL monomer structure" /></td>
<td><img src="image" alt="PCL polymer structure" /></td>
</tr>
</tbody>
</table>
Table 1.2. Physico-chemical properties of important biodegradable polymers (157).

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Tm (°C)*</th>
<th>Tg (°C)*</th>
<th>Modulus (Gpa)*</th>
<th>Degradation (months)**#</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGA</td>
<td>225-230</td>
<td>35-40</td>
<td>7.0</td>
<td>6 to 12</td>
</tr>
<tr>
<td>PLLA</td>
<td>173-178</td>
<td>60-65</td>
<td>2.7</td>
<td>&gt;24</td>
</tr>
<tr>
<td>PDLLA Amorphous</td>
<td>55-60</td>
<td>1.9</td>
<td>12 to 16</td>
<td></td>
</tr>
<tr>
<td>PCL</td>
<td>58-63</td>
<td>(-65)-(-60)</td>
<td>0.4</td>
<td>&gt;24</td>
</tr>
<tr>
<td>PDL-LGA (85/15)</td>
<td>Amorphous</td>
<td>50-55</td>
<td>2.0</td>
<td>5 to 6</td>
</tr>
<tr>
<td>PDL-LGA 75/25</td>
<td>Amorphous</td>
<td>50-55</td>
<td>2.0</td>
<td>4 to 5</td>
</tr>
<tr>
<td>PDL-LGA (65/35)</td>
<td>Amorphous</td>
<td>45-50</td>
<td>2.0</td>
<td>3 to 4</td>
</tr>
<tr>
<td>PDL-LGA (50/50)</td>
<td>Amorphous</td>
<td>45-50</td>
<td>2.0</td>
<td>1 to 2</td>
</tr>
</tbody>
</table>

*Depends on molecular weight and distribution.

**Time to complete mass loss. Rate also depends on sample geometry.
1.5.2.2. Polylactide (PLA)

Polylactide, or called poly(lactic acid) (PLA), is another important biodegradable poly(α-hydroxyl acid). Like PGA, PLA can be synthesized from lactic acid or lactide through bulk polymerization. Lactide is the cyclic diester of lactic acid. Ring-opening polymerization of lactide produces a high molecular weight material. Lactic acid, known as milk acid, has two optical isomers, D and L. L-lactic acid exists in the human body and plays a role in normal metabolism and exercise. Commercial lactic acid is usually produced by bacterial fermentation.

Based on the chiral nature of lactic acid, PLA exists in poly-L-lactide (PLLA) resulting from the polymerization of L.L-lactide, and in poly-D,L-lactide (PDLLA) resulting from the polymerization of a mixture of L- and D-lactides. PLLA is a semicrystalline polymer with approximately 37% crystallinity, a glass transition temperature of between 50 and 80°C, and a melting temperature in the area of 150 to 178°C. It exhibits high tensile strength, high modulus, and a lower degradation rate, which make it more suitable for load-bearing applications such as in orthopaedic fixations and sutures. PDLLA is an amorphous polymer with lower tensile strength, higher elongation, and a higher degradation rate, which make it more attractive as a drug delivery carrier.

1.5.2.3. Poly(ε-caprolactone) (PCL)

The most outstanding feature of PCL is its low glass-transition temperature near -60°C, which gives it greater elongation at room temperature. PCL is a semicrystalline polymer with a low melting temperature between 59 and 64°C. Ring-opening polymerization of ε-caprolactone yields PCL of high molecular weight. This polymer is often used to improve the mechanical properties and processability of other polymers by copolymerization or by blending. The low cost of PCL also makes it very attractive for biomedical applications. The degradation rate of PCL is significantly slower than that of other primary biodegradable polymers (usually more than 2 years), making it well suited for the design of long-term implantable devices.
such as Capronor (158), an FDA-approved contraceptive device, and long-term controlled drug delivery systems.

1.5.2.4. Poly(lactide-co-glycolide) (PLGA)

The copolymerization of lactide with glycolide yields PLGA, a copolymer that reduces both the crystalline degree and the stiffness of PGA and improves the hydrophilicity of PLA. PLGA offers a wide range of degradation rates, from several weeks to years, depending on the lactide/glycolide ratio. For example, PLGA (LA/GA, 50/50) yields a fast degradation rate and may degrade completely after only several weeks. PLGA (10/90) was developed as a suture (trade name Vicryl) that can be absorbed within 3-4 months (157). In addition to being applied as sutures, PLGA has been widely studied and used in other biomedical applications.

1.5.3. Biodegradation

A material is biodegradable when it has an unstable and weak linkage site (or chemical bond) in its molecular chain under specific conditions. In the case of biodegradable aliphatic polyesters, the weak site is the ester bond. Their degradation is an intrinsic hydrolysis process (159-162), in which water penetrates in the bulk of the polymer device and chemically attacks the ester bond, resulting in the breakdown of molecular chains capped by the –OH and –COOH groups. This step leads to the molecular weight reduction prior to mechanical property reduction and mass loss. There are two types of degradation: bulk degradation and surface erosion. The aliphatic polyesters degrade through the bulk, which is typically catalyzed by the internally confined acidic degradation products (autocatalysis). In the case of hydrophobic polymers, such as polyanhydrides and polyorthoesters (163), surface erosion occurs, which is particularly useful in controlled drug release.

Material degradation is influenced by a variety of factors, such as chemical composition and structure, molecular weight and distribution, crystalline degree,
morphology, and size. Furthermore, environmental factors including temperature, pH (164), and enzyme action (165, 166) also affect degradation rate.

1.5.4. Applications

1.5.4.1. Artificial substitutes (167-169)

The first well-known application for biodegradable polymers is the absorbable artificial suture (Table 1.3). Various suture products of different mechanical strength and degradation rate have been produced. Biodegradable polymers have also been applied in orthopaedic fixation devices (pins, rods, screws, tacks, ligaments), dental applications (guided tissue regeneration), cardiovascular surgery (stents, grafts), and abdominal procedures (intestinal anastomosis rings).

1.5.4.2. Controlled drug release system

The goal of controlled drug release is to maintain drug concentration within a continuously effective range over a long period in a physiological environment and to achieve an optimal use of drugs by eliminating the systemic side effect and the effect of under/overdosing. Drugs are normally released from biodegradable carriers according to a predesigned protocol. Ideally, a drug delivery system must be biocompatible, comfortable for the patient, capable of carrying a sufficient quantity of drugs, easy to administer and remove, and easy to fabricate and sterilize. Biodegradable polymers, such as PLGA, are particularly interesting because of their biocompatibility, adjustable degradation rate, and absorbability of their degradation products (170-174). Biodegradable materials are commonly fabricated into microspheres, nanospheres, or simply into a coating. The immobilized drugs within the materials are released into the physiological environment following degradation (163).

1.5.4.3. Tissue engineering

Table 1.3 lists the products made of biodegradable polymers. See also Section 1.3 “Tissue engineering”.
Table 1.3. List of commercial biodegradable devices (165).

<table>
<thead>
<tr>
<th>Application</th>
<th>Trade Name</th>
<th>Composition</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sutures</td>
<td>Dexon</td>
<td>PGA</td>
<td>Davis and Geck</td>
</tr>
<tr>
<td></td>
<td>PGA Suture</td>
<td>PGA</td>
<td>Lukens</td>
</tr>
<tr>
<td></td>
<td>Vicryl</td>
<td>PGA-LPLA</td>
<td>Ethicon</td>
</tr>
<tr>
<td></td>
<td>Monocryl</td>
<td>PGA-PCL</td>
<td>Ethicon</td>
</tr>
<tr>
<td></td>
<td>Polysorb</td>
<td>PGA-LPLA</td>
<td>U.S. Surgical</td>
</tr>
<tr>
<td>Interference screws</td>
<td>Sysorb</td>
<td>DLPLA</td>
<td>Synos</td>
</tr>
<tr>
<td></td>
<td>Arthrex</td>
<td>LPLA</td>
<td>Arthrex</td>
</tr>
<tr>
<td></td>
<td>Bioscrew</td>
<td>LPLA</td>
<td>Linvatec</td>
</tr>
<tr>
<td></td>
<td>Phusiline</td>
<td>LPLA-DLPLA</td>
<td>Phusis</td>
</tr>
<tr>
<td>Suture anchor</td>
<td>Bio-Statak</td>
<td>LPLA</td>
<td>Zimmer</td>
</tr>
<tr>
<td>Anastomosis clip</td>
<td>Lactasorb</td>
<td>LPLA</td>
<td>Davis and Geck</td>
</tr>
<tr>
<td>Anastomosis ring</td>
<td>Valtrac</td>
<td>PGA</td>
<td>Davis and Geck</td>
</tr>
<tr>
<td>Dental</td>
<td>Drilac</td>
<td>DLPLA</td>
<td>THM Biomedical</td>
</tr>
<tr>
<td>Angioplastic plug</td>
<td>Angioseal</td>
<td>PGA-DLPLA</td>
<td>AHP</td>
</tr>
<tr>
<td>Screw</td>
<td>SmartScrew</td>
<td>LPLA</td>
<td>Bionx</td>
</tr>
<tr>
<td>Pins and rods</td>
<td>Resor-Pin</td>
<td>LPLA-DLPLA</td>
<td>Geistlich</td>
</tr>
<tr>
<td>Tack</td>
<td>SmartTack</td>
<td>LPLA</td>
<td>Bionx</td>
</tr>
<tr>
<td>Plates and mesh</td>
<td>LactoSorb</td>
<td>PGA-LPLA</td>
<td>Lorenz</td>
</tr>
<tr>
<td>Guided tissue</td>
<td>Antrisorb</td>
<td>DLPLA</td>
<td>Atrix</td>
</tr>
<tr>
<td></td>
<td>Resolut</td>
<td>PGA-DLPLA</td>
<td>W.L. Gore</td>
</tr>
</tbody>
</table>
1.6. Proposed project

Regulating cellular activity is vital to cells and tissues to maintain normal biological functions. Extensive work has been imposed to control cellular adhesion, migration, proliferation, gene expression, and protein secretion. As a physical cue, electrical stimulation (ES) is effective in regulating cellular functions. Indeed, ES has shown clinical benefits in healing wounds, in treating disease, and in enhancing neuronal regeneration. To successfully apply ES in tissue engineering to control cellular functions, namely, to localize the electrical field within a conductive nerve guidance channel to enhance nerve regeneration, a functional biomaterial that is both conductive and biodegradable must be designed.

Combined with specific surface properties, such as reversible oxidation-reduction and ion-exchange properties, as well as biocompatibility both in vitro and in vivo, conductive polypyrrole (PPy) has been used in a variety of biomedical applications such as biosensors and controlled drug release. Furthermore, ES through a conductive PPy-coated plate has been shown to enhance neurite outgrowth (31). These findings therefore confirm the unique potential of PPy in tissue engineering. Despite the high degree of conjugation, which makes PPy rigid and unprocessable, this aspect can be improved by combining it with flexible polymers to form PPy composites. In the literature, most PPy composites were combined with non-biodegradable polymers and used primarily in conventional industrial applications.

Biodegradable aliphatic polyesters, such as polylactide (PLA), polyglycolide (PGA), and their copolymers, have been widely used in tissue engineering because of their recognized biocompatibility, adjustable degradation rate, and relatively wide-spectrum mechanical properties. Thus, combining PPy with aliphatic polyesters, such as PLA, is expected to generate a biomaterial that is both conductive and largely biodegradable. Ideally, this substrate should be able to be processed into specific forms, namely, 3-D porous structures and nerve guidance channels. Because PPy is not biodegradable, the amount of PPy in the composite should be as low as possible.
Moreover, to study cellular responses to ES through a degradable bioconductor, an appropriate electrical cell-culture plate must be designed. The electrical stability of these conductive composites in a physiological environment must also be validated. Most importantly, the efficacy of the bioconductor-mediated ES on cellular activity must be investigated.

1.7. Description of project

1. Objectives

The general objective is to regulate cell activities by applying ES through conductive and biodegradable substrates (bioconductors). The strategies include the preparation of bioconductors by combining electrically conductive polypyrrole (PPy) with biodegradable poly(hydroxy acid) such as polylactide (PLA), the design of an electrical cell-culture plate, and the investigation of cell responses to ES through bioconductors. Following five specific objectives are included:

(1) To prepare and characterize PPy/PLA bioconductors;
(2) To design an appropriate electrical cell-culture plate;
(3) To study bioconductor stability in a physiological environment;
(4) To study bioconductor biocompatibility;
(5) To investigate the effects of ES through bioconductors on cellular activities, such as cellular adhesion, viability, proliferation, gene expression and protein secretion.

2. Hypotheses

(1) A PPy/PLA substrate with low PPy content can maintain sufficient electrical stability in a cell culture environment and has accepted biocompatibility.
(2) Electrical stimulation applied through bioconductors used as cell culture substrates can modulate certain cellular behaviours.
3. Approaches

(1) Synthesize PPy by an emulsion polymerization, fabricate PPy/PLA membranes by one-step and two-step methods, and characterize the physical-chemical properties of PPy/PLA bioconductors;

(2) Design an appropriate electrical cell-culture plate integrated into a computerized controlling/monitoring system;

(3) Study electrical stability of bioconductors in cell culture medium using a home-made electrical cell-culture system;

(4) Culture fibroblasts on bioconductors and test cytotoxicity;

(5) Culture fibroblasts on bioconductors with or without ES, and characterize cell morphology, adhesion, viability, proliferation, gene expression and cytokine secretion.
CHAPTER II

MATERIALS and METHODS
2.1. Preparation of the conductive PPy/PLA membranes

2.1.1. Method I: one-step *in situ* polymerization

Pyrrole (98%, Aldrich Chemical Co., Milwaukee, WI, USA) was distilled twice under reduced pressure and stored in a refrigerator at 4°C prior to use. PLA (120–150 kDa, Hycail B.V., Noordhorn, The Netherlands) was purified using a chloroform-methanol system and dried under vacuum for 2 weeks. For the emulsion polymerization of the PPy, a small amount of pyrrole and aqueous sodium dodecyl sulfate (SDS, 99%, Fluka Chemie GmbH, Steinheim, Switzerland) solution were added to a PLA solution in CHCl₃. FeCl₃ dissolved in H₂O was introduced dropwise into the mixture under vigorous stirring to trigger an oxidative polymerization. The molar ratio of FeCl₃ to pyrrole was 2.3 and the weight ratio of pyrrole to PLA was 5% and 20%. The polymerization continued at room temperature for 4 h, followed by precipitation with ethyl alcohol and a wash in deionized water. The membranes were obtained by casting the precipitated PPy/PLA composite onto a PTFE plate and vacuum-dried for 2 weeks. The PPy/PLA membranes with different PPy contents (wt%) were obtained by diluting the prepared PPy/PLA (20%) solution with pure PLA solution, followed by the same casting and drying procedures. The resulting PPy/PLA and PLA membranes were 0.3 mm in thickness.

2.1.2. Method II: two-step polymerization

PPy particles were firstly synthesized using a water-in-oil emulsion system by adding aqueous dodecylbenzenesulfonic acid sodium salt (DBS, Aldrich Chemical Co.) solution to chloroform, followed by a specific amount of pyrrole. The oxidative polymerization was initiated by adding FeCl₃ (FeCl₃: pyrrole = 2.3:1) to the emulsion under vigorous stirring, and was maintained at room temperature for 18 h. Then the PPy particles were washed repeatedly to remove any remaining emulsifier and FeCl₂. Secondly, the PPy/PLA membranes (5-10%, w/w) were prepared by mixing PPy particles with PLA solution in chloroform, followed by solution casting and solvent evaporation. The thickness of the PPy/PLA membranes was approximately 0.5 mm.
2.2. Physical-chemical characterization

2.2.1. Scanning electron microscopy (SEM)

The morphology at the surface and cross-section of the PPy/PLA composite membranes was observed with Jeol JSM 35 scanning electron microscopy at an accelerating voltage of 15 kV. Prior to observation, the specimens were spatter-coated with gold. SEM analyses were performed on all PPy/PLA membranes.

2.2.2. X-ray photoelectron spectroscopy (XPS)

The surface elemental composition of the specimens was analyzed using a Perkin Elmer PHI model 5600 X-ray photoelectron spectrometer (Eden Prairie, MN, USA). A standard magnesium source (1253.6 eV) was used to obtain the low-resolution survey spectra. The photoelectrons emitted from the sample surface under X-ray excitation were collected at a take-off angle of 45° and analyzed with a hemispheric electron energy analyzer operating at pass energy of 187.9 eV (survey scans) or 5.85 eV (high-resolution scans). During the analysis, the base chamber pressure was in the order of $10^{-10}$ Torr. The high-resolution spectra were curve-fitted using the software provided by the manufacturer. XPS analyses were performed on all PPy/PLA membranes.

2.2.3. Surface electrical resistivity

The surface resistivity $R_s$ (Ω/square) of the specimens was measured according to the AATCC Test Method 76-1995 (175). Briefly, two parallel rectangular flat copper electrodes were firmly placed on the surface of the specimen. A DC voltage $V$ (volt) was applied between the electrodes and the current $I$ (ampere) was recorded. The surface resistivity $R_s$ (Ω/square) was then calculated using the following formula:
\[ Rs = \frac{V W}{I D} \]  

(1)

where \( W \) is the width of the specimen and \( D \) the distance between the two electrodes. Each measurement was performed in duplicate.

### 2.2.4. Electrical stability in cell culture medium

The PPy/PLA membranes containing 5% PPy were cut into specimens 2.5x3.0 cm\(^2\) in size, and these specimens were assembled on the bottom of a homemade electrical cell culture plate. The two longitudinal edges of each specimen were firmly pressed against two separated platinum (99.99%) electrodes connected to a power source. Eagle's minimum essential medium (MEM) (M-0268, Sigma Chemical Co., St. Louis, MO, USA) was then added into the plate, along with 0.05% sodium azide to prevent bacterial growth. There was no direct contact between the electrodes and the medium. Incubation was carried out in a standard cell culture incubator at 37°C for a period of 1000 h without changing medium. A constant DC potential gradient of 5.0±0.1 mV/mm was applied to the specimen and the change of electrical current was monitored through a computerized data-collecting system. The sensitivity of the current measurement was 1 nA. Pure PLA membrane and PPy-coated poly(ethylene terephthalate) (PET) fabrics (Milliken Research Co., Spartanburg, SC, USA) were used as references under the same experimental conditions. The surface resistivity of the conductive fabrics was 280 \( \Omega \)/square at room temperature. The choose of pure PLA, i.e., an insulator made of the same base polymer as the PPy/PLA composite, was to demonstrate if the experiment set-up was free of ionic current. The PPy-coated PET fabric, on the other hand, was the only other PPy/polymer composite that has been tested for biomedical applications (127, 128).
2.3. Cytocompatibility assay

2.3.1. Fibroblast culture

Human skin fibroblasts (Clonetics, San Diego, CA) were washed twice with Dulbecco's modified Eagle (DME) medium, seeded in $1.0 \times 10^4$ cells/75 cm$^2$ flasks and grown in DME medium supplemented with 100 UI/ml penicillin G (Sigma-Aldrich Canada Ltd, Oakville, ON, Canada), 25 μg/ml gentamicin (Schering, Pointe-Claire, QC, Canada), and 10% fetal calf serum (FCS) (Gibco, Burlington, ON, Canada). The medium was changed three times a week. When the cultures reached 90%, the cells were detached from the flasks using a 0.05% trypsin-0.1% EDTA solution, washed twice, and resuspended in DME-supplemented medium at a final concentration of $10^6$ cells/ml. The cells were then used to seed the biomaterials under study.

2.3.2. Membrane sterilization

The sample membranes were sterilized by ethylene dioxide gas at 37°C according to standard industrial procedures in the facility of the Saint-François d'Assise Hospital, and stored under vacuum for at least two days to remove the possible residual gas.

2.3.3. Cell attachment on the bioconductors

The PPy/PLA membranes were first incubated in DMEM for 24 h under cell culture conditions in order to move residual chemicals that may possibly remained in the membranes after PPy synthesis. Fibroblasts were seeded at $20 \times 10^4$/specimen. After 4 h, the cells were trypsined and counted using trypan blue exclusion test (n=3, see below). Tissue culture Petri dishes (35 mm diameter) seeded with the same number of cells were used as reference. After 24 h, the cells were fixed and observed by SEM (see below).
2.3.4. Cell viability and proliferation on the bioconductors

Fibroblasts were seeded on the PPy/PLA membranes (6.0 \times 10^4 \text{ cells/cm}^2, or 17 \times 10^4 \text{ cells/specimen}) and cultured for 4 and 6 days, respectively. The entire culture medium (2 ml) was refreshed every 24 hours. Cells were assayed by fluorescent Hoechst staining and SEM. The cell viability was tested by MTT (n=6, see below). Tissue culture Petri dishes seeded with the same cell density were used as control group. The experiment was repeated four times.

![Diagram](image)

**Figure 2.1.** A representative well of the home-made multi-well electrical cell culture plate.
2.4. Design of multi-well electrical cell culture plates

Eight-well electrical cell culture plates were made from poly(methyl methacrylate) (PMMA) plates. Each well measured $2.6 \times 2.0$ cm$^2$. Rectangular PMMA blocks were fabricated with thin platinum electrodes attached to fix the membranes to the bottom of the wells and to maintain pressure on the electrodes against the membranes. The blocks were tightly inserted into the wells to ensure sufficient electrical contact between electrode and membrane, as can be seen in Figure 2.1. After inserting the blocks, the test surface exposed to the culture medium reduced to $2.0 \times 1.4$ cm$^2$. The electrodes, 0.1 mm in thickness, were connected to a power source (Topward 3303A, Topward Electric Instruments Co. Ltd., Taipei, Taiwan, China) capable of supplying either constant direct current (DC) or constant potential. The DC current passing through the specimens was individually recorded by means of a computerized multi-channel data acquisition system.

2.5. Electrically stimulated cell culture on bioconductors

2.5.1. The range of working current

The PPy/PLA membranes were fixed at the bottom of the homemade electrical cell culture plates and connected with electrodes as described in Section 2.4. The membranes assembled in plates were sterilized with EO gas at 37°C following standard industrial procedures. Before cell seeding, the membranes were first incubated in DMEM for 24 h under cell culture conditions to clean the membranes. Fibroblasts were seeded ($5.0 \times 10^3$ cells/cm$^2$, or $2.0 \times 10^4$ cells/specimen) onto the test membranes and were cultured for 48 h in normal condition, i.e., without electrical stimulation, followed by a 4-day culture under electrical stimulation. Figure 2.2 illustrates how cells were electrically stimulated on a conductor. The culture medium was refreshed each day. At the end of the culture period, the cells were either stained with a fluorescence dye for photographing or trypsinized for cell viability and growth evaluation.
Various direct electrical currents were realized through a low voltage DC power source (Topward 3303A) working at a constant potential and various sliding resistances. Following initial DC current density were used: 0, 0.7, 1.3, 6.7, 13.3, 26.7, 53.3, and 106.7 \( \mu \text{A/mm}^2 \). Because the resistivity of the membranes increased with time during cell culture, the electrical potential applied to each membrane was adjusted every 24 h in order to maintain the initial current. The stimulation currents were recorded using the computerized data-collecting system. This experiment was repeated 4 times.

For each electrical stimulation experiment, fibroblasts were also cultured on cell culture Petri dishes for a period of 6 days. The dishes were observed daily under an inverted microscope and at the end of the 6 days were detached for cell growth assessment.

**Figure 2.2.** Cells were grown and electrically stimulated on a bioconductor.
2.5.2. ES uplimit on cell functions

To test the working window or upper limit of ES tolerable to fibroblasts, fibroblasts (2.0 × 10^4 cells/cm^2, or 17 × 10^4 cells/specimen) were cultured on the bioconductors for 24 h and stimulated by various DC potential gradients ranging from 0 to 500mV/mm. After 24-hour stimulation, cells were fixed for analysis (total 48 hours of culture) or were allowed to grow for another 24 hours (total 72 hours of culture) for analysis. The experiment was repeated 3 times.

2.5.3. ES on cell viability

2.5.3.1. Cells cultured electrically on bioconductors

Fibroblasts (6.0 × 10^4 cells/cm^2, or 17 × 10^4 cells/specimen) were seeded on the PPy/PLA membranes. For the electrically stimulated (ES) test, a constant DC potential gradient (100 mV/mm) was applied immediately following cell seeding. Fibroblasts were exposed to this electrical field for 1, 2, 4, and 24 h. As control, fibroblasts were cultured at the same seeding density on the nonelectrically stimulated conductive specimens (non-ES). In all of the experiments (n=5), fibroblasts were also cultured in tissue culture Petri dish (1.7 × 10^4 cells/cm^2, or 17 × 10^4 cells/plate) only to demonstrate the normal growth of the same passage of cells under standard conditions.

2.5.3.2. Effect of electrical field conditioned medium on cell growth

To test the potential effect of leachables from the PPy/PLA membranes following ES, 16 PPy/PLA membranes were assembled in the multi-well electrical culture plates, preincubated 48 h in culture medium, and incubated in fresh medium for 24 h in the presence (n=8) or absence (n=8) of a constant DC potential gradient (100 mV/mm). Following incubation, the culture medium (1.5 ml) was collected and used to culture fibroblasts in tissue culture Petri dishes at 20.0 × 10^4 cells/plate for 48 h without changing the medium. MTT was performed at the end of the culture period. Four tissue culture Petri dishes were used as controls.
2.5.3.3. Cell culture on the insulating surface in the presence of ES

To test the potential effect of weak ionic current possibly generated between the two Pt electrodes, eight plastic plates were cut from the tissue culture Petri dishes, cleaned and assembled in the multi-well electrical cell culture plates. Four of these plates were used in the presence of a constant DC potential gradient (100 mV/mm) while the others were used in the absence of DC potential gradient as controls. Cells were seeded at $6.0 \times 10^4 / \text{cm}^2$ and kept cultured for 24 h, after which time MTT assay was performed. Cells were also cultured on tissue culture Petri dish at $17 \times 10^4 / \text{plate}$ as reference. The experiment was repeated 4 times.

2.5.3.4. Cell culture on the gold-coated PD plates

To assay the effect of electrical current, the tissue culture Petri dishes (PD) were cut into $2.6 \times 2.0 \text{ cm}^2$ plates, cleaned and sputter-coated with gold in the sputter coater used to prepare SEM specimens. The cytotoxicity of the gold-coated PD plates was firstly tested using the multi-well electrical cell culture plates. Fibroblasts were seeded at $20 \times 10^4 / \text{membrane (or plate)}$. After 4 h, the cells were trypsined and counted using trypan blue exclusion test (see below). Tissue culture Petri dishes (35 mm diameter) seeded with the same number of cells were used as reference.

The gold-coated PD plates were assembled in the multi-well electrical culture plates. Two sets of experiments were carried out. In the first set, a constant DC current density $2.5 \mu \text{A/mm}$ (because of the extremely thin coating of gold, the thickness of the gold layer was ignored and the current was divided only by the width of the plate that was 20 mm) was applied across the surface of the cells cultured for 2, 4, and 24 h. The fibroblasts were seeded at $6.0 \times 10^4 \text{ cells/cm}^2$. This experiment was repeated four times. In the second set, cells were seeded at $2.0 \times 10^4 \text{ cells/cm}^2$ and cultured for 96 h under constant DC current density of 2.5, 25.0, and 250.0 $\mu \text{A/mm}$, respectively. The electrical potential gradient was recorded as 0.025, 0.25, and 2.5 mV/mm, respectively. Light microscopic photos were taken and an MTT assay was performed following each culture period. This experiment was performed four times.
2.5.4. ES on cell proliferation

Fibroblasts \((6.0 \times 10^4 \text{ cells/cm}^2, \text{or } 17 \times 10^4 \text{ cells/specimen})\) were seeded on the PPy/PLA membranes and cultured for 24 hours. Then a constant, small DC potential gradient (ES) of \(50 \text{ mV/mm}\) was applied to cells through the two ends of the membranes for another 24 hours, using the homemade electrical cell culture system, followed by an additional 48-hour culture (total 4 days, assigned as ES 4d) or a 96-hour culture (total 6 days, ES 6d) without ES. Cells cultured on the conductors without ES (control 4d and control 6d) and on the tissue culture Petri dish (PD) served as controls. The entire culture medium was refreshed every 24 hours. The flowchart of the experiment is as follows:

Both the pH and temperature of the culture medium were monitored on selected wells, and no measurable changes were found during the entire period of cell culture. The experiment was repeated four times. Following these experimental design we performed different analyses as described in the sections below.
2.5.5. ES on cytokine expression and secretion

Fibroblasts were seeded and cultured on the PPy/PLA membranes in the absence or presence of ES (same as Section 2.5.4.). Every 24 hours, 2.0 ml of the culture medium was collected and renewed. The collected medium was stored at -80°C for an ELISA test.

2.6. Cell assessment

2.6.1. Trypan blue test

At the end of the cell culture, fibroblasts were detached from the membranes following treatment with 0.05%-trypsin-0.01 EDTA solution for 10 min at 37°C. After, the cells were washed twice with DMEM supplemented culture medium and then centrifuged. The pellet obtained from centrifuge was re-suspended in 1 ml of culture medium and used to determine cell viability and total cell number using the trypan blue exclusion test (176). To do this, 100 μl of fibroblast cell suspension was mixed with the same volume of trypan blue solution and the mixed solution were incubated in ice for 5 min. Then the total number of cells and the number of viable cells were counted using a hemacytometer. The cell count was performed in triplicate for each cell suspension and three cell suspensions were counted at least. The results were reported as means±SD of all cell suspensions.

2.6.2. Cell visualization by fluorescent Hoechst staining

Immediately after cell culture, cells on the sample membranes were washed three times with PBS then incubated with 2 μg/ml Hoechst 33342 (H42) (Riedel de Haen; Seele, Germany) in PBS for 15 min at room temperature before being washed and mounted with a coverslip in 50% glycerol mounting medium. Stained cells were observed and photographed using an epifluorescence microscope (Axiophot, Zeiss, Oberkochen, Germany). Representative pictures were presented.
2.6.3. Ultrastructural analyses using SEM

The cells attached to the membranes were fixed with 4% paraformaldehyde in PBS at room temperature for 15 min. The fixed cells were washed in distilled water followed by dehydration in graded ethanol solutions (from 50 to 100%). The specimens were further dried in a vacuum oven at room temperature and sputter-coated with gold for SEM analysis. The morphology on the surface of the specimens was investigated using SEM. Representative pictures were presented.

2.6.4. Cell viability test by MTT assay

Immediately following each culture period (see section 2.5.4), a 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay (MTT-assay) was used to measure the viability of the cells. In brief, a 200-μl MTT solution (5 mg/ml in PBS) was added to each culture well that contained the test membrane and the 2 ml cell culture medium. The cultures were then kept at 37°C for 4 h. At the end of the 4 h incubation, the supernatant was removed, and the 2 ml HCl (0.04 N) in isopropanol was added to each well; the incubation then continued for another 15 min. Finally, 200 μl of solution was transferred in triplicate from each well to a 96-well plate, and the optical density of the solutions was read at 550 nm using an ELISA-Reader (model 680, BioRad Laboratories, Mississauga, ON, Canada). The MTT test determines cell metabolic activity by measuring the ability of the mitochondrial succinate-tetrazolium reductase to convert the yellow compound MTT into a blue formazan dye. The amount of dye produced was proportional to the number of live metabolically active cells. The experiment was repeated four times.

2.6.5. IL-6 and IL-8 gene activation by RT-PCR assay

Total cellular RNA was extracted from the cells cultured with or without ES, using Qiagen RNeasy Mini kits (Qiagen, Valencia, CA, USA). The mRNA was reversely transcribed into cDNA using Maloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and random hexamers (Amersham
Pharmacia Biotech, Inc., QC, Canada). One microliter of each cDNA product was added to 24 µl of the PCR mixture that contained Taq polymerase (Qiagen) with forward and reverse specific primers (Table 2.1) (Biosource, Montreal, QC, Canada). All reactions were performed in a MyCycler thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was measured in each sample as a positive control to confirm the integrity of the RT-PCR. The RT-PCR conditions for IL-6, IL-8, and GAPDH were as follows: 95 °C for 2 min; 35 cycles at 94 °C for 1 min, 54 °C for 45 sec, and 72 °C for 1 min; and a final extension at 72 °C for 10 min. After the PCR, 4 µl samples were loaded on 1.5% agarose gels containing loading buffer. The electrophoresis was run at 110 V for 45 min. The gels were photographed under UV light, and the relative intensities of the bands were measured on digitalized images using the public domain NIH Image program. This experiment was repeated at least three times. Quantitatively, the mRNA expression of cells on PD was defined as 1, and the values of the ES and control groups were calculated relative to the PD group.

Table 2.1. Primer sequences used for the RT-PCR.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Direction</th>
<th>Sequences</th>
<th>Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>Forward</td>
<td>5'-TCAATGAGGAGACTTGCTG-3'</td>
<td>260 bp</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-GATGAGTTGTCATGTCTGC-3'</td>
<td></td>
</tr>
<tr>
<td>IL-8</td>
<td>Forward</td>
<td>5'-TTG GCA GCC TTC CTG ATT-3'</td>
<td>260 bp</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-GATGAGTTGTCATGTCTGC-3'</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward</td>
<td>5'-AAC TTC TCC ACA ACC CTC TG-3'</td>
<td>220 bp</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-TCTCGCTCTGGAAGATGCTG-3'</td>
<td></td>
</tr>
</tbody>
</table>
2.6.6. IL-6 and IL-8 secretion by ELISA

The concentrations of IL-6 and IL-8 secreted in supernatants were quantified using a quantitative, sandwich enzyme immunoassay technique. Cell culture supernatants were centrifuged and assayed using an enzyme-linked immunosorbent assay kit (ELISA, R&D Systems, Minneapolis, MN, USA). The 96-well microplate was read at 450 nm and analyzed using a Model 680 Microplate Reader (Bio-Rad Laboratories). According to the manufacturer, the ELISA kits can detect less than 0.35 pg/ml of IL-6, and 0.97 pg/ml of IL-8.

2.7. Statistical analysis

Experiments were performed at least three times or as indicated in each section. Experimental values are given as means ± SD. The statistical significance of the difference between the control and test values was evaluated using a One-Way ANOVA. Results were considered significant at $p < 0.05$. 
3.1. Characterization of the PPy/PLA bioconductor

3.1.1. One-step *in situ* polymerization

3.1.1.1. Morphology

Figure 3.1A shows the SEM observations, which revealed the presence of many irregular micro-domains on the surface of the PPy/PLA membranes. A high magnification image (Figure 3.1B) shows that these micro-domains were formed by the PPy nanoparticles that were entangled with a small amount of PLA. These nanoparticles were spherical, 50–200 nm in diameter, and formed aggregations dispersed in the PLA matrix. Cracks and pores of micro or sub-micro size were readily identified in the PPy domains. PPy particles were also found in the smooth region where apparent PPy aggregations had not formed (Figure 3.1C). At the cross-section of the PPy/PLA membranes, the PPy nanoparticles had formed clusters in the membrane (Figure 3.2A); these clusters extended to the surface (Figure 3.2B), forming the surface-exposed PPy domains, as indicated by the arrows in Figure 3.2B.

The surface morphology of the PPy/PLA membranes also changed with the PPy content, as is shown in Figure 3.3. With the increase in PPy content, the number of the surface-exposed PPy domains not only augmented, but also formed a continuous network, which eventually covered the entire surface.

The morphology at the cross-section of the PPy/PLA membrane after 1000 h stability test exhibited many micropores from less than 1 μm to a few micrometers in size (Figs. 3.4C and D), which were rarely found in virgin membranes (Figs. 3.4A and B). These micropores were resulted from the degradation of the PLA material, a phenomenon that also identified in previous studies (177). These micropores were formed preferentially under the non-porous skin of the membrane.
Figure 3.1. SEM photomicrographs at the surface of a PPy/PLA membrane containing 5% PPy, showing the PPy domains (A), the aggregation of PPy nanoparticles in the domains (B), and the dispersion of non-aggregated PPy nanoparticles in the PLA matrix (C).
Figure 3.2. SEM photomicrographs at the cross-section of a PPy/PLA membrane containing 5% PPy, showing the PPy nanoparticle clusters (A) and the surface-exposed domains as the extension of the clusters to the surface (B), as indicated by the arrows.
Figure 3.3. SEM photomicrographs on the surface of the PPy/PLA membranes with various PPy content: (A) 5%; (B) 7%; (C) 9%; (D) 17%.
Figure 3.4. SEM photomicrographs at the cross-sections of the PPy/PLA membrane experienced 1000 h of electrical stability test (C, D) and the virgin PPy/PLA membrane (A, B), showing the micropores formed under the skin of the tested membrane.
3.1.1.2. XPS

Table 3.1 presents the surface elemental composition of the representative PPy/PLA membrane and PPy-coated PET fabrics. It was found that the percentage of nitrogen on the surface of the non-incubated PPy/PLA membrane containing 5% PPy was 4.0%, compared to 11.4% for the PPy-coated PET. Incubation raised 13% and 18% oxygen on PPy/PLA membrane and PPy-coated fabric, respectively. The chlorine found on the non-incubated fabric disappeared after incubation.

<table>
<thead>
<tr>
<th>Specimens</th>
<th>[C]</th>
<th>[O]</th>
<th>[N]</th>
<th>[Cl]</th>
<th>[Si]</th>
<th>[Ca]</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPy/PLA</td>
<td>62.9</td>
<td>32.8</td>
<td>4.0</td>
<td>---</td>
<td>0.3</td>
<td>---</td>
</tr>
<tr>
<td>(before incubation)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(after incubation)</td>
<td>58.3</td>
<td>37.1</td>
<td>4.0</td>
<td>---</td>
<td>0.6</td>
<td>---</td>
</tr>
<tr>
<td>PPy-coated fabrics</td>
<td>68.7</td>
<td>18.4</td>
<td>11.4</td>
<td>1.4</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>(before incubation)</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(after incubation)</td>
<td>64.6</td>
<td>21.8</td>
<td>13.2</td>
<td>---</td>
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<td>0.4</td>
</tr>
</tbody>
</table>

High resolution XPS spectrum is used to identify positively charged and non-positively charged nitrogen atoms in PPy. A high portion of positively charged nitrogen normally indicates a high conductivity. As shown in Figure 3.5A, the high resolution N$_{1s}$ spectrum of PPy (PLA contains no nitrogen) in the virgin PPy/PLA membrane showed one major peak and a distinct shoulder at the high binding energy
side. It was then curve-fitted into two component peaks and assigned to pyrrolylium nitrogen (−NH−) at 399.7 eV and positively charged nitrogen (−NH+−) atoms at 401.7 eV (178). Based on the surface areas of the two peaks, the ratio of the oxidized-to-neutral nitrogen atoms on the surface of the PPy/PLA membrane was calculated to be 2.23 (69/31), compared to 0.19 (16/84) for the same ratio on the PPy-coated PET fabrics (Figure 3.6A). Following incubation in MEM for 1000 h, the N1s peak lost the shoulder and simultaneously acquired a small tail on the lower binding energy side of the major peak, rending the N1s profile asymmetric. This peak was again curve-fitted into two components; the first component peak was assigned to the main −NH− at 399.7 eV, and the second to the deprotonated imine nitrogen (=N−) at 399.2 eV (Figure 3.5B). The assignment of the tail to =N− was somehow arbitrary, as the shift was only 0.5 eV, which was much smaller than the reported values of 1.9 eV (178) and 1.8 eV (179). The percentage of the deprotonated imine atoms was 39.2%. For the PPy-coated fabrics incubated in MEM for 700 h (Figure 3.6), the deprotonated imine nitrogen atoms occupied 48.2%. For both materials, −NH+− became undetectable after incubation.

3.1.1.3. Surface electrical resistivity

Figure 3.7 shows the relationship between the PPy content in the PPy/PLA membranes and their surface resistivity. The resistivity exhibited a typical percolation behaviour, with a threshold at approximately 3%. At this relatively low threshold, the resistivity recorded as low as 1×10³ Ω/square. At this stage, PPy content below 3% resulted in a dramatic increase in resistivity. Above 9%, the decrease of resistivity was very moderate. Between 1% and 17% of PPy content, the resistivity varied from 2×10⁷ to 15 Ω/square, a variation of six orders of magnitude.
Figure 3.5. XPS N$_{1s}$ spectra of a PPy/PLA membrane containing 5% PPy: (A) before incubation; (B) after a 1000-hour incubation in MEM and subjected to 5.0 mV/mm.
Figure 3.6. XPS N$_{1s}$ spectrum of the PPy-coated PET fabrics: (A) before incubation; (B) after incubation in MEM and subjected to 5.0 mV/mm.
Figure 3.7. Relationship between the surface electrical resistivity of the PPy/PLA membranes and the PPy content (n=2).
Figure 3.8. Relationship between electrical current and incubation time in the electrical test carried out in a MEM solution.
3.1.1.4. *In vitro* electrical stability

During the stability test, the potential $V$, the distance of the two parallel electrodes $D$, and the width of the specimen $W$ were constant values. According to formula 1, the change in the current ($I$) inversely reflects the change of resistivity ($R_s$). As can be seen in Figure 3.8, for the PPy/PLA membrane, the current sharply increased from 264 to 274 $\mu$A in the first few minutes with the addition of MEM, followed by a rapid decline to approximately 250 $\mu$A at 8 h. It then slowly decreased to 210 $\mu$A at 100 h, 110 $\mu$A at 400 h and 40 $\mu$A at 1000 h, which was 80%, 42% and 15% of the initial value, respectively. The current-decreasing rate was approximately 1.8 $\mu$A/h in the first 8 h, and 0.2 $\mu$A/h thereafter. For the PPy-coated fabrics, the current increased from 294 to 331 $\mu$A in the first few minutes following the addition of MEM, then quickly decreased to 16 $\mu$A at 100 h and 0.3 $\mu$A at 400 h, meaning 5% and 0.1% of the initial value, respectively. In the first 100 h, the current-decreasing rate for the PPy-coated fabrics was approximately 2.8 $\mu$A/h. For the pure PLA membrane, (an insulator), no current was detected during the entire experiment, signifying no ionic current between the electrodes.

3.1.2. Two-step method

The morphology of the PPy particles is presented in Figure 3.9. The particles were mostly round in shape and formed clusters. The size of the particles was rather uniform and was about 100-300 nm that was slightly larger than that prepared by the one-step method. The PPy/PLA membrane was composed by interconnected PPy networks dispersed in PLA matrix.
Figure 3.9. Scanning electron photomicrograph of the PPy particles.
3.2. The PPy/PLA bioconductor was nontoxic to fibroblasts

The microscopic observations revealed that after 4 h, the human cutaneous fibroblasts adhered to the PPy/PLA (Figure 3.10A) surfaces and were well distributed. Cell adhesion and viability were confirmed by the trypan blue exclusion test. As is shown in Figure 3.10B, 4 h after cell seeding, the fibroblasts adhered to the PPy/PLA and were viable, which confirms the nontoxicity of this membrane to human fibroblasts. Identical findings were obtained with the gold-covered surface, on which adherent fibroblasts displayed a significant number of viable cells after a 4-h culture (Figure 3.11).

After 24 h of cell culture on the bioconductor, fibroblasts attached and spread well (Figure 3.12A) on the conductive PPy/PLA membranes and started to divide at 24 hours (Figure 3.12B, arrows).

Fluorescent staining (Figure 3.13A) revealed that fibroblasts proliferated well on the PPy/PLA membranes after 4 days of cell growth. At 6 days, the surface of the bioconductor was fully covered by cells (Figure 3.13B). SEM (Figure 3.13C and D) demonstrated that the fibroblasts were highly elongated and showed a dense nucleus. MTT (Figure 3.14) indicated that fibroblasts cultured on the bioconductors proliferate more than that of the PD group at 4 days. At 6 days, fibroblasts proliferation was comparable in both groups. These results therefore confirm that the PPy/PLA bioconductor was nontoxic to the human cutaneous fibroblasts and are suitable to study the ES effect on human fibroblasts.
**Figure 3.10.** Fibroblasts on the tissue culture Petri dish (PD) and the conductive PPy/PLA membranes at 4 hours, showing no significant difference in the distribution (A) and number (B) (n=3). The cells on the PPy/PLA surface were stained with Hoechst.
Figure 3.11. Fibroblasts on the tissue culture Petri dish (PD) and the gold-coated tissue culture Petri dish (Gold-PD) at 4 hours, showing no significant difference in the distribution (A) and number (B) (n=3).
Figure 3.12. SEM photomicrographs of fibroblasts attached on PPy/PLA membranes at 24 hours with spindle shape (a) and at the dividing phase (b). Arrows show the dividing nuclei. The results indicate that the bioconductors support fibroblasts attachment and spreading.
**Figure 3.13.** Fibroblasts attached on PPy/PLA membranes stained with Hoechst at 4 days (A) and 6 days (B), and observed by SEM at 4 days (C) and 6 days (D).
Figure 3.14. Viability of fibroblasts cultured on the PPy/PLA bioconductor for 4 (n=6) and 6 days (n=6).
3.3. Electrically stimulated cell culture on bioconductors

3.3.1. The range of working current

Figure 3.15 shows the effect of DC current stimulation on cell growth. It is evident that the plates experienced medium range current stimulation, i.e., initial current density at 1.33 and 6.67 μA/mm², recorded a significantly higher number of viable cells compared with the other plates. At both low and high current conditions, the growth of cells was similar to that without electrical stimulation (0 μA). This phenomenon was also confirmed by the fluorescence observations (Figure 3.16). The number of viable cells on Petri dishes was $76 \pm 20 \times 10^4$/ml, which was higher than that on the PPy/PLA membranes. The relatively low number of cells on the PPy-containing polymers with respect to that on the cell culture dishes was also observed in previous studies (127, 180) and was caused by the low initial cell adhesion onto the PPy-containing polymers.

The recorded stimulation current curves of one of the experiments were presented in Figure 3.17. Because of the fine adjustment of electrical potentials on each culture plate, most of the currents maintained at their initial levels, except the highest current set at 106.7 μA/mm², where the decrease of conductivity was relatively significant and so was the current adjustment.

3.3.2. ES uplimit on cell functions

Fluorescent staining (Figure 3.18B) and SEM (Figure 3.18D) showed that after 24-hour ES (total 48 hours of culture) at 100 mV/mm, fibroblasts normally scattered, attaching themselves to the conductive membrane and showing typical spindle-like morphology similar to that of the control and PD groups. Similar observations were found at 72 hours (data not shown). However, after 24-hour ES at 500 mV/mm, the cells were mostly round and formed aggregations (Figure 3.18A). The SEM revealed various cell morphologies, including both spindle and round shapes, as well as broken cell membranes with many particulates (Figure 3.18C). After another 24-hour culture
without ES (total 72 hours of culture), nearly all cells died and detached themselves from the bioconducors (data not shown).

**Figure 3.15.** The effect of DC current stimulation on cell growth, showing that medium range current intensity up-regulated cell proliferation. Significant difference between single and double labelled data pairs (p < 0.05, n=3).
Figure 3.16. Fibroblasts stained with fluorescence on their nuclei, showing that medium range DC current stimulation induced cell growth on the PPy/PLA membranes.
Figure 3.17. The recorded DC current curves over the entire cell culture period. The stability was obtained through a fine adjustment of the potential applied to each culture plate.
Figure 3.18. Fluorescent Hoechst staining and SEM photomicrographs of the fibroblasts attached on the PPy/PLA membranes after 24-hour ES at 500 mV/mm (A, C) and 100 mV/mm (B, D). Fibroblasts appeared round and aggregated (A, arrows) with cell membrane apparently dissolved (C, arrows) at 500 mV/mm. At 100 mV/mm, cells had normal appearance (B) and showed classic spindle morphology (D).
3.3.3. ES upregulated cell viability

3.3.3.1. ES produced no cytotoxic leachables

As can be seen in Figure 3.19A, the media collected from the ES and non-ES membranes generated MTT values of 0.355 and 0.375, respectively, compared to 0.312 for the tissue culture Petri dishes. While the small differences between each pair of data sets were significant ($p < 0.05$, D.F. = 15), it is clear that the culture media conditioned by either ES or non-ES membranes did not contain cytotoxic leachables.

3.3.3.2. Multi-well electrical culture plate generated no strong ionic current to affect cell viability

In the experiment using insulating plastic plates, the ionic current density in culture medium possibly generated between the two Pt electrodes was 0-2.5 $\mu$A/mm$^2$ when a 2 V voltage was applied between the electrodes. This small ionic current had no effect on cell viability (Figure 3.19B). No statistical difference was found between ES and nonelectrical stimulation (non-ES).

3.3.3.3. Constant electrical field stimulation promoted the proliferation of fibroblasts seeded on the PPy/PLA membranes

The shape of the cells with or without ES was comparable throughout the four observation periods. Representative observations are presented in Figure 3.20. Under both conditions at 24 h, the fibroblasts were elongated and showed a dense nucleus (more visible on the ES membranes). Slightly higher cell density was observed on the ES membranes compared to the nonstimulated PPy/PLA membranes (Figure 3.20A). The MTT test results (Figure 3.20B) show that as early as the second hour post-ES, the MTT values of the cells were significantly higher on the ES membranes than on the non-ES membranes ($p < 0.05$, D.F. = 8). This significant difference was also maintained 24 h post-culture ($p < 0.01$, D.F. = 8). The cell viability on the ES membranes was 2 and 4 times of that on the non-ES membranes at 2 and 24 h.
respectively. Interestingly, the fibroblasts appeared to have adhered and proliferated better on the PPy/PLA membrane with ES than on the tissue culture Petri dishes. SEM analysis (Figure 3.21) confirms the adhesion and initial proliferation (24 h) of the cutaneous fibroblasts on the PPy/PLA membranes. By 24 h, these membranes (and specifically, the electrically stimulated ones) were completely covered by fibroblasts. The cells also migrated into the porous structure of the membranes (Figure 3.21B). The cells were elongated and displayed a small nucleus and reduced cytoplasm, which confirms that they were not under stress.

3.3.3.4. Fibroblast adhesion and proliferation on the gold-coated PD plates were not modulated by constant electrical current

As is shown in Figure 3.22A, fibroblasts adhered and adopted normal cell morphology on the gold-coated tissue culture Petri dishes with or without the presence of 2.5 μA/mm ES. The cells were distributed uniformly throughout the surface with an apparently similar density. The MTT assay (Figure 3.22B) recorded no difference for the gold-coated specimens with or without ES at each incubation period. Figure 3.23 further demonstrates the ineffectiveness of DC current on the cells on the gold-coated surfaces, showing no phenotypic difference between the stimulated and nonstimulated fibroblasts. These cells exhibited an elongated shape with a reduced nucleus and cytoplasm. Following MTT analysis, cell viability (Figure 3.23B) also remained unchanged on the gold surfaces regardless of the presence of a wide range of strong DC currents. The overall results therefore indicate that at the various intensities and culture periods, cutaneous fibroblasts growth on the gold-coated membranes were in no way hindered by the DC current.
Figure 3.19. Fibroblast viability after 24 hours of culture in a medium previously used to incubate PPy/PLA membranes showed no cytotoxic leachables in the medium (A). The weak ionic current between the two Pt electrodes had no effect on cell viability after 24 hours (B) (n=8).
Figure 3.20. Fibroblasts on the PPy/PLA membranes at 2 and 24 hours with or without ES. Note the comparable cell distribution and high cell density on the ES membranes (A). Panel (B) shows a significantly higher cell viability on the membranes with ES (100 mV/mm) (n=5).
Figure 3.21. SEM photomicrographs showing fibroblasts attached (2 hours), spread (4 hours) and proliferated (24 h) identically on PPy/PLA surfaces with or without ES (100 μV/mm). A virgin membrane is shown as (A). (B) reveals the fibroblasts migrated into the microporous structure of the membranes at 4 hours.
Figure 3.22. Fibroblasts on the gold-coated tissue culture Petri dish (Gold-PD) for 2 and 24 hours with or without ES, showing that DC current at 2.5 μA/mm had no effect on cell attachment, distribution and morphology (A), and no significant impact on cell viability (B) (n=3).
Figure 3.23. Fibroblasts on the gold-coated tissue culture Petri dish for 96 hours with or without ES at various surface current densities, showing that a wide range of DC current density had no effect on cell morphology (A) and viability (B) (n=4).
3.3.4. ES upregulated cell proliferation

Fluorescent staining (Figure 3.24) revealed that fibroblasts proliferated well on the PPy/PLA membranes in the presence or absence of ES. For the ES group at 4 days, the number of cells appeared to be slightly higher than that seen on the control membranes, and the cells formed a cell sheet (Figure 3.24C, inset). At 6 days, the surface of the membranes was fully covered by cells for both the ES (Figure 3.24B) and control groups (Figure 3.24D); no significant difference in cell numbers was seen. The SEM demonstrated that ES at 50 mV/mm strength did not modify the cell morphology, which appeared equally elongated and similar in size for both the ES and control groups. The bioconductor membranes of the ES group were completely covered by the cells at both 4 and 6 days (Figure 3.25).

As shown in Figure 3.26, ES significantly increased cellular viability, with an MTT value 25% higher than that of the control group at 4 days (p < 0.05, D.F. = 10). At 6 days, when cells reached confluence in all three groups, ES-induced cells showed only a slightly higher viability, which was insignificant (p > 0.1). At 6 days, the viability of the cells cultured on the control bioconductors (MTT = 0.63) was comparable to that on the PD (0.62), which confirmed that the conductive PPy/PLA membranes had a comparable cytocompatibility to that of the PD. The comparable results at 6 days were to be expected because of the similar cell density and viability at confluence.
Figure 3.24. Fluorescent Hoechst staining of the fibroblasts proliferated on the control (A and B) and ES at 50 mV/mm (C and D) PPy/PLA membranes for 4 and 6 days. Arrow points at a cell sheet formed on the ES membranes at 4 days (C). The results show that the bioconductors supported cell proliferation in the presence or absence of ES and that at 6 days ES does not significantly affect cell number.
Figure 3.25. SEM photomicrographs of fibroblasts cultured on the Control (A and B) and ES (C and D) PPy/PLA membranes for 4 and 6 days, showing the complete cover of cells on the bioconductor membranes of the ES group at both 4 and 6 days. The particulate surface morphology of the underneath membranes remains visible.
Figure 3.26. Viability of fibroblasts cultured on the PPy/PLA membranes with (50 mV/mm) or without ES for 4 (n=6) and 6 days (n=6). The results indicate that ES mediated by bioconductors significantly enhanced cell viability at 4 days.
3.3.5. ES enhanced expression of IL-6 and IL-8

Figure 3.27A shows that ES significantly enhanced the IL-6 mRNA expression of the cells cultured on the bioconductors. Quantitatively, the IL-6 expression of the ES group was 78% and 70% higher than that of control group at day 4 (p < 0.01) and day 6 (p < 0.05), respectively (Figure 3.27B).

For IL-8 mRNA expression (Figure 3.27C), at 4 days, ES through the bioconductors generated a significant increase of 60% compared to that of control group (p < 0.01). At 6 days, however, the expression of the ES group was only slightly higher (10%) than the control group (p > 0.1).

3.3.6. ES enhanced secretion of IL-6 and IL-8

The ELISA results, as shown in Figure 3.28A, demonstrated that the concentration of IL-6 secreted by the cells cultured on the PPy/PLA membranes (control) was 39 pg/ml at 4 days and 35 pg/ml at 6 days. As a reference, the cells cultured on PD secreted IL-6 of 72 pg/ml at 4 days and 65 pg/ml at 6 days. However, ES mediated by bioconductors dramatically increased IL-6 secretion, with a concentration of 222 pg/ml at 4 days and 381 pg/ml at 6 days. This represents an increase of 4.7 fold (p < 0.05, D.F. = 8) at 4 days and 9.9 fold at 6 days (p < 0.05, D.F. = 8), respectively.

Using ES mediated through the bioconductors also significantly enhanced the IL-8 secretion (p < 0.05, D.F. = 8), as shown in Figure 3.28B. The ES-induced IL-8 production was 1177 pg/ml at 4 days, compared to the control group’s production of 718 pg/ml. At 6 days, the IL-8 secretion of the control group decreased to 102 pg/ml, while it remained at 1019 pg/ml for the ES group, representing a nearly 10-fold increase. There was no detectable IL-8 secretion in the supernatant of the PD group.
**Figure 3.27.** Expression of IL-6 (A, B) and IL-8 (A, C) with (50 mV/mm) or without ES at 4 and 6 days, showing significantly increased IL-6 and IL-8 mRNA expression at 4 days. The results indicate that ES mediated by a bioconductor can dramatically upregulate fibroblasts gene expression.
Figure 3.28. Secretion of IL-6 (A) and IL-8 (B) with (50 mV/mm) or without ES, showing dramatic increase of IL-6 and IL-8 production following ES. The results demonstrate that ES mediated by a bioconductor can significantly upregulate cell secretion.
4.1. Preparation and characterization of the PPy/PLA bioconductor

4.1.1. Morphology

Based on the SEM observations, the PPy/PLA composite was a two-phase system in which the conductive PPy particles dispersed in the insulating PLA continuous phase. Rather than uniformly dispersed, the PPy nanoparticles largely presented themselves as microdomains of various sizes located both on the surface and in the membrane. A model may be used to describe the structure of the PPy/PLA composite. Initially, the PPy nanoparticles formed aggregations; these aggregations then constituted microdomains; the microdomains further connected to each other to form a network embedded in the PLA. At high PPy content, these microdomains became large and interconnected, forming an interpenetrating network (IPN) with the PLA. The conductivity of the composite is therefore expected to depend on the properties of the PPy network, including the conductivity of the PPy particles, the contact between the particles, the size of the domains, the extent of network development, and the number of surface-exposed PPy domains which have a vital role in interfacing the PPy network with the external circuit. While a certain degree of PPy particle aggregation is unavoidable if the goal is to achieve conductivity at very low PPy content, a significant aggregation above micrometer scale may cause non-uniform cell adhesion on the membrane. In addition, PPy particle aggregation on the surface is also expected to cause variation on surface conductivity as function of position on the surface. However, this heterogeneity in conductivity is at micrometer scale (Figure 3.1A) and discontinuity in conductivity, if any, would be at sub-micrometer scale (Figure 3.1C). It is important to notice that the apparent smooth area in Figure 3.1A does contain PPy particles and is not insulator. In light of this, nevertheless, further improvement in material homogeneousness, preferably to sub-micrometer scale, will be beneficiary to avoid potential non-uniform cell adhesion and heterogeneous electrical stimulation to cells.
4.1.2. Electrical stability

The electrical stability of synthetic conductive polymers in the presence of water remains a challenge. Environmental or aging stability studies have been mainly limited to the thermal stability in air or in water vapour (134, 150). In a recent effort, water was eventually prevented by using ionic liquid to enhance the electrical stability of conductive polymers (181). In biomedical applications, however, the presence of water is normally unavoidable. Previous study revealed that PPy underwent dedoping and deprotonation under the synergistic action of water and current (177), which has been further confirmed in this experiment. While the deterioration of PPy conductivity in water appears inevitable at this time, for practical purposes, the goal here was to achieve and maintain a biologically meaningful DC current, 10–200 μA for example (31), during a period of 1–2 months or longer. The drop of conductivity over time, which is certainly undesirable, would complicate the interpretation of experimental data but is not anticipated to be harmful to cells or host tissues. Because the biodegradable composite is destined for use in vivo, and with PPy regarded as non-degradable, the challenge for a bioconductor is to reach a relatively stable and sufficient conductivity at the lowest possible PPy concentration in a physiological environment. As matter of fact, as a biodegradable composite, there is no need for infinite conductivity. The membrane experienced 1000 h electrical stability test already showed large amount of micropores caused by hydrolytic degradation. The development of such degradation will eventually destroy the physical structure hence the electrical conductivity of the membrane. Nevertheless, the new PPy/PLA composite successfully maintained its conductivity at $3 \times 10^3 \Omega/$square after 1000 h in MEM, and thereby accomplished the goal of the present study. The PPy/PLA composite membrane thus provides a useful platform for our electrical stimulation study.

During the stability test, it was found that the current in both the PPy/PLA membrane and PPy-coated PET fabrics increased sharply in the first few minutes following the addition of the MEM. In previous report (177), a transient polarizing current occurred between two electrodes upon the addition of the medium. In this study, however, as
the platinum electrodes had no direct contact with the aqueous medium, no polarizing or ionic current occurred, as confirmed by the control experiment using pure PLA membrane. This initial increase of current should therefore only be caused by the interaction between the PPy and the aqueous medium. It was suggested that additional electrical paths had established among the neighbouring superficial PPy particles as a result of replacing air with water. Following the initial increase, the current began to decrease in the PPy/PLA membranes and the PPy-coated PET fabrics. This was caused by the dedoping and deprotonation in the presence of water, as supported by the XPS results in this experiment as well as in previous report (177). Because the PPy particles in the PPy/PLA membrane were located both on the surface and in the membrane, the rapid decrease of current in the first 8 h was likely related to the PPy on the surface, and the slow decline thereafter was hence associated with those PPy particles located inside the PLA membrane. Therefore, the latter was probably controlled by the diffusion of water in the PLA. On the other hand, the ready access of water to the PPy coating on the PET fabrics was responsible for the rapid deterioration of conductivity accompanying the PPy-coated fabrics. Compared to previous study (177) in which the current varied initially and then stabilized, the PPy/PLA membranes used in this experiment behaved differently. In fact, the conductive membranes used in the two studies actually displayed very different PPy particle distributions. This then confirms that the preparation method and morphology of a PPy/polymer have a significant impact on its electrical stability.

4.1.3. Comparison of two methods

The PPy/PLA nanocomposites were prepared by two approaches: one-step and two-step methods. In the one-step in situ polymerization, PPy particles were synthesized directly in a PLA solution. While this procedure does have several advantages, including excellent dispersion, it is time-consuming to wash out emulsifiers and FeCl₂. Therefore, the other approach, called two-step method, in which PPy particles were synthesized separately prior to being mixed with PLA solution, was developed to prepare the PPy/PLA composite. This approach enabled us to thoroughly wash the PPy particles to reduce the level of impurities. As a new synthetic route, the PPy/PLA
composites were tested for cytotoxicity towards human cutaneous fibroblasts. The results clearly indicate that the PPy/PLA material was not cytotoxic. The fibroblasts attached and proliferated very well on the PPy/PLA surface. It was also clearly demonstrated that this type of bioconductor was as effective as tissue culture Petri dish for supporting cell adhesion, spreading, and proliferation. This method was applied to perform subsequent studies.

4.2 Electrically stimulated cell culture on bioconductors

4.2.1. ES on cell adhesion and growth

In the case of anchorage-dependant cells such as fibroblasts, adhesion is the first step in the interaction with a substrate and is essential before cells are able to spread and divide. Cell adhesion to nonbiological surfaces such as synthetic polymers is normally mediated by serum proteins such as fibronectin (FN) in culture medium. High FN adsorption to PPy under ES has been reported and correlated with enhanced neurite outgrowth (182). In that work however, pure PPy was used as a working electrode, which differs significantly from the setup used in the present experiment, in which PPy/PLA composite was used as a semiconductor in a closed circuit environment. In another work where electrical leads were directly connected to a PPy film on opposite ends and carried DC current of 50 μA, like in our case, ES was found to significantly reduce the absorption of bovine serum albumin and fibrinogen to PPy film (183). Our results show that a constant electrical field strength of 100 mV/mm induced significantly greater cell viability/proliferation than that shown by the nonstimulated cells cultured on the same substrate of identical surface morphology and chemistry. Surprisingly, the fibroblasts with ES tended to display greater proliferation compared to those cultured in the tissue culture Petri dishes (Figure 3.20B). The tissue culture plates were not intended to be compared with the conductive membranes because of different surface areas and seeding densities. Instead, these plates were used only to show the normal function of fibroblasts under the standard conditions. Cell viability readings in tissue culture plates were normally
higher than that in conductive membranes, except for the ES membranes. Due to the
difficulty experienced in trypsinizing all of the cells out of the PPy/PLA membranes
at 24 h, the number of cells shown in Figure 3.20 remains unknown. Figure 3.20A,
however, does indicate greater cell density on those membranes with ES, although
this slightly higher number is unlikely to explain the major difference shown in
Figure 3.20B. We therefore conclude that a constant electrical field of 100 mV/mm
has upregulated the mitochondrial activity of human cutaneous fibroblasts and also
probably enhanced their adhesion. The relatively large standard deviation to the ES
membranes likely reflects the heterogeneous nature of the ES caused by the
nonuniform distribution of PPy particles on membrane surface. Further work needs to
be done to improve the homogeneity of PPy distribution.

In the literature, both electrical field and current were reported to be effective in
modulating cell behaviors (14, 182). In the capacitive or salt-bridge type of setup,
these parameters are actually related, as the conductivity of the culture medium is
relatively constant. In the system used in the present study, however, the two
parameters can be independently controlled by changing the conductivity or PPy
content of the conductive membrane. To determine the importance of current density
in ES, we used highly stable and conductive surfaces, namely, gold-coated plates.
Surprisingly, a wide range of surface current density (from 2.5 to 250 μA/mm) had no
effect on cell adhesion or cell viability. Figure 3.15 demonstrated that a DC current
intensity between 0.67 and 106.7 μA/mm² was shown to affect fibroblasts differently.
The difference between these two results is in fact the substrate. To generate identical
current density across the PPy/PLA and gold surfaces, the required electrical field
differed because of the difference in conductivity of the two materials. The required
potential gradient was much lower for the gold-coated plates, because of their low
resistance (in the order of 10 Ω). A logical conclusion is that it is electrical field or
potential gradient that plays the more significant role. This shall not however rule out
the importance of current when potential gradient reaches a certain level.
As a precaution, two control experiments were performed to rule out the possible interference of leachables and ionic current. Even after a thorough wash, impurities may continue to leach out of a membrane during an experiment, particularly when electricity is applied. These impurities, primarily emulsifier and ferric dichloride, may interfere with cell behavior. Our control experiments revealed that leachables, if any, did not affect cell viability (Figure 3.19A). The second control experiment showed that electrodes on the insulating substrates did not generate enough biologically meaningful ionic current in culture medium to affect cell behavior. This means that the observed cellular reaction to ES was solely because of the electrical field applied through the PPy/PLA conductive substrate. Both control experiments were important to validate our findings.

4.2.2. ES on cytokine expression and secretion

To study the effect of ES on cytokine secretion, we measured IL-6 and IL-8 production in fibroblast culture supernatant. The ELISA results showed that the ES mediated by the PPy/PLA bioconductors dramatically enhanced the secretion of IL-6 and IL-8. Indeed, the ES-induced release of both IL-6 and IL-8 increased nearly 10-fold. Again, four days after ES, when cells had already reached confluence in all three groups, the concentration of IL-6 and IL-8 remained much higher in the ES group than in the other two groups, meaning that the mean IL-6 and IL-8 production by individual cells was significantly upregulated by ES. Thus, ES mediated by a bioconductor provides a highly efficient approach to regulating cellular cytokine production. IL-6 is a multifunctional pro-inflammatory cytokine that is involved widely in wound healing (184) and in the inflammatory and immune responses initiated by infection or injury (185). IL-6 is also known to help differentiate myeloid stem cells (186) and to stimulate hepatocytes (187). Also, IL-8 is an important chemokine involved in wound healing, with a primary role of attracting immune cells like neutrophils to the site of inflammation (188). IL-8 is also a well-known, potent player in angiogenesis (189). Clearly, regulating IL-6 and IL-8 is significant in wound healing and is likely to be useful in promoting tissue regeneration by rapidly recruiting inflammatory cells and promoting angiogenesis in scaffolds.
This new approach to enhance cytokine production may be generalized to much broader scenarios; for instance, it could be used to promote the production of bioactive proteins such as interferons. Several interferons -- for example, interferons \( \alpha-2a \) and interferons \( \alpha-2b \) -- have been shown to have pharmaceutical effects and have already been used in clinics to treat such immune-system-related diseases as leukemia (190) and such virus-caused infections as chronic hepatitis C (191). ES mediated by conductive substrates may provide a new avenue of pursuit in biotechnology.

In this study, the IL-6 mRNA expression showed an increase of 70% with ES. It is unlikely, however, that this increase in gene expression, can explain the near 10-fold difference in cytokine secretion. One logical hypothesis is that inducing ES through the bioconductors significantly enhanced the activity of translation enzymes. Note that the IL-8 expression showed similar results. At 6 days, no significant difference in IL-8 mRNA expression was found between the ES and control groups, while IL-8 secretion was significantly higher in the supernatant. This hypothesis is also supported by Koyama’s report (24) that ES through a 3-electrode electrochemical reaction system induced the NGF production of astroglial cells. Koyama proposed that the ES-induced NGF release might be associated with the \( \text{Ca}^{2+} \)-dependent PKC mediation pathway via the \( \text{Ca}^{2+} \) channel. Since ES have significant effects on IL-6 and IL-8 expression and secretion, and since fibroblast proliferation was modulated by ES, it would be important to study the other bioactive molecules in the presence of ES, such as adhesion molecules (fibronectin, cadherin, etc.) and growth factors (FGF, EGF, etc).

### 4.2.3. Mechanism of ES on cell functions

Just how ES interacted with fibroblasts is not at all clear. Cell membranes represent extremely complex, balanced electrical states that feature quasi-electrostatic surface charges of membrane proteins and electrodynamic ionic fluxes in and out of cell membrane channels, including those that are voltage-gated. Any disturbance of these
delicate states electrically -- for example, using ES, as in this study -- can be expected to “trigger” cell reactions. However, the interactions between membrane receptors and soluble messenger molecules such as growth factors can be viewed as the interaction of electrical fields between specific chemical groups. Our hypothesis is that ES induced a conformational change of membrane proteins, thereby triggering a cascade of cell reactions. Cell death at the high field strength of 500 mV/mm may have been caused by irreversible membrane damage. Electrical stimulation has shown its capacity to modulate cell membrane permeability (192) and cytoskeletal structure (23). Furthermore, reduced cell membrane fluidity was reported during ES (23). Apparently, these ES-induced effects are related to cell membrane structures. Further study is clearly required to elucidate the underlying mechanisms.

In this study, it is found that the working window of ES can be as high as 100 mV/mm and that ES at both 50 and 100 mV/mm did not affect cell morphology. In addition, we did not detect any measurable pH or temperature variation during the period of cell culture, in the presence or absence of ES. These findings indicate that the cell responses to ES observed in this present work were indeed the consequence of an electrical field mediated by the bioconductors. Also, studies on other cell types such as keratinocytes, endothelial cells could be of great importance.

4.3. Perspectives

This study on electrically conductive polymer composites is based on the hypothesis that such composites can be used to host the growth of cells so that electrical stimulation can be applied directly to the cells through the composite to modulate cellular behaviours. The electrically stimulated cell culture results presented in this thesis proved this hypothesis and consequently validated this new biomaterial. This could be potentially highly significant to the development of biomaterials because the electrically conductive biodegradable polymers represent a new category of functional biomaterials that are yet to be explored. Compared with traditional
electrical stimulation with electrodes, the electrical stimulation applied through a scaffold made of conductive polymer is confined in the boundary of the scaffold, therefore can be much more precisely controlled. In addition to fabricating conductive scaffold that has direct implication in tissue engineering, such biocompatible and implantable electrical conductor may also be useful in the area where an integration of microelectronics and biological tissue is required.
CHAPTER V

CONCLUSIONS
This thesis reports on the following: the design and preparation of a novel degradable bioconductor; the design and preparation of a multi-well electrical cell-culture plate to host electrically stimulated cell cultures and the monitoring of electrical parameters; the study of the electrical stability of this novel bioconductor in a physiological environment; and the study of cell responses to electrical stimulation (ES) using this degradable bioconductor. The following conclusions were obtained.

A degradable bioconductor with adjustable conductivity was successfully prepared. With a PPy content of as low as 5wt%, this bioconductor showed acceptable electrical stability and cytocompatibility similar to that observed in a tissue culture Petri dish. Compared to other ES methods using electrodes, degradable bioconductors have many advantages: (i) ES is predominately localized on the surface and within the bioconductor; (ii) Electrical potential gradient can be controlled by changing the bioconductor's resistivity; (iii) Bioconductors can be bioactivated to provide a multifunctional surface for cell growth; and (iv) Bioconductors can be easily prepared in 3-D structures such as conductive nerve guidance channels. This type of material therefore displays significant potential in tissue engineering and bioelectronic applications.

A multi-well electrical cell culture device was designed and fabricated to apply ES to cell-cultured bioconductors. Cells were cultured on a bioconductor connected to an enclosed electrical circuit. The electrical current through the circuit was recorded and processed. Compared to other ES devices, such as those based on salt bridges and metallic or indium-tin oxide (ITO), the ES device experimented in this study prevented the metal electrodes from direct contact with the culture medium and basically eliminated the oxidation-reduction reaction on the electrodes. The effect of ionic current on cellular activity was also negligible using this novel device.

Regulating cellular activity temporally and spatially is essential in order to regenerate tissue of normal architecture and biological function. This process is heavily mediated by various kinds of biological factors, including inflammatory cytokines.
Therapeutically significant cytokines have already been investigated for the treatment of malignant and infectious diseases. In this thesis, a novel approach – ES mediated by degradable bioconductors – was developed and trialed to effectively regulate such cell functions as morphology, adhesion, viability, proliferation, gene expression, and protein secretion. These findings highlight, for the first time, the potential of a potent and effective technique that could be used to regulate tissue regeneration in conductive scaffolds through ES-modulated cytokine secretion and also to increase cytokine productivity for biotechnological applications.
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APPENDIX I Protocol of RNA extraction

1. Principle

Cells are lysed and homogenized in 2-mercaptoethanol solution to release RNA and inactivate RNases. Lysates are spun through a filtration column to remove cellular debris and shear DNA. The filtrate is then applied to a high capacity silica column (spin column) to bind total RNA, followed by washing and elution.

2. Reagents based on Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA, USA, Cat. No. 74104) and QIAshredder (Cat. No. 79654)

- Sterile, RNase-free pipet tips
- Microcentrifuge (with rotor for 2 ml tubes)
- Disposable gloves
- QIAshredder Mini Filtration columns
- RNeasy Mini Spin Columns (pink)
- Collection tubes (1.5 ml)
- Collection tubes (2 ml)
- 2-mercaptoethanol 14.3 M (2-ME)
- Buffer RLT
- Buffer RW1
- Buffer RPE (concentrate)
- RNase-Free Water
- Ethanol (96-100)

Notes
- Maximum binding capacity 100 µg RNA
- Maximum loading volume 700 µl
- RNA size distribution RNA >200 nucleotides
- Minimum elution volume 30 µl
- Maximum amount of cells 1 x 10^7
- Sample (cells grown on a membrane in 2.0 x 1.7 cm^2 area)
- Wear gloves at all times

3. Preparation of working solution

- 70% ethanol
  70 ml ethanol (96-100%) + 30 ml H_2O (DEPC treated)
- RPE washing buffer
  11 ml RPE (concentrate) + 44 ml ethanol (96-100%)
- 2-ME working solution (1%)
  20 µl + 20 ml RLT buffer
4. Procedure (Modification from Qiagen RNeasy Mini Kit)

(1) Culture cells on the membrane (2.0 × 1.7 cm²). Remove the supernatant carefully transfer the cells/membrane into a 6-well Petri dish.
(2) Lyse cells by adding 200 µl 2-ME working solution (1%); pipet to mix.
(3) Pipet the lysate directly into a QIAshredder filtration column placed in a 2 ml collection tube.
(4) Centrifuge for 2 min at 10⁴ rpm.
(5) Add 200 µl 70% ethanol to the homogenized lysate, and mix well by pipetting.
(6) Transfer the solution to an RNeasy spin column placed in a 2 ml collection tube. Close the lid gently, and centrifuge for 2 min at 10⁴ rpm. Remove the solution.
(7) Add 700 µl buffer RW1 to the RNeasy spin column. Close the lid gently, and centrifuge for 30 s at 10⁴ rpm. Remove the solution and change a new 2 ml collection tube.
(8) Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently, and keep for 5 min. Centrifuge for 30 s at 10⁴ rpm. Remove the solution.
(9) Repeat step 9.
(10) Place the RNeasy spin column in a new 1.5 ml collection tube. Add 30 µl RNase-free water directly to the spin column membrane. Close the lid gently, and keep for 10 min.
(11) Centrifuge for 1 min at 10⁴ rpm.

Remove the RNeasy spin column. Close the lid gently, and store it at −80°C.

![Image of Quality of RNA samples by SDS-PAGE](image)

Figure A1. Quality of RNA samples by SDS-PAGE
APPENDIX II Protocol of RNA qualification

1. Principle

Samples like RNA with different molecular weight have different migration speed in gel at specific electrical field. By comparing with standard molecular weight marker, RNA can be confirmed by the band position.

2. Reagents

- Agarose
- TBE
- Loading buffer (LB)
- Molecular weight marker (Mw) (1 Kb DNA ladder, Life Technologies AG, Basel, Switzerland)
- RNA sample
- Tubes (0.5 ml)

3. Preparation of working solutions

- Agarose (1.5%)
  1.2 g agarose + 80 ml H$_2$O
- TBE 1X
  10 mM Tris + 1 mM EDTA in DEPC H$_2$O (pH 7.5)
- Mw solution
  2 µl Mw standard + 8 µl H$_2$O
- RNA sample test solution
  2 µl RNA sample + 8 µl H$_2$O

4. Procedures

(1) Prepare the agarose gel at room temperature for 30 min.
(2) Prepare the sample and Mw solution.
(3) Put the gel into TBE solutions.
(4) Add 2 µl LB to each sample and Mw solution, respectively.
(5) Transfer sample and Mw solution to the gel.
(6) Run at 110 volts for 45 min
(7) Take photographs by UV light.

5. Example

Shown as Figure A1.
APPENDIX III Protocol of RNA quantification

1. Principle

RiboGreen regent can bind RNA and shows bright sensitive green fluorescence. There is a linear relationship between fluorescence and RNA concentration. The RNA concentration can be calculated by reading the fluorescence in a standard fluorescent microplate reader. The sensitivity is ranged from 1 ng/ml to 1 μg/ml.

2. Regents

- RiboGreen RNA quantitation kit (Molecular Probes Inc., Eugene, OR, Cat. No. R-11490)
- Black 96-well microplates
- TE buffer (10 mM Tris, 1 mM EDTA pH 7.5)
- FL600 fluorescent microplate reader with excitation filter at 485 nm and emission filter at 530 nm
- 16S and 23S ribosomal RNA standard (from E. coli), in TE buffer (100 μg/ml)

3. Preparation of working solution

- TE IX
  10 mM Tris + 1 mM EDTA in DEPC H₂O (pH 7.5)
- RiboGreen solution (1/200)
  100 μl RiboGreen reagent + 19.9 ml TE
- Sample solution (shown as Table A1)

Table A1. Preparation of sample solution for RNA quantification.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dilution</th>
<th>RNA (μl)</th>
<th>H₂O (DEPC, μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1/1000</td>
<td>1</td>
<td>499</td>
</tr>
<tr>
<td>2</td>
<td>1/3000</td>
<td>1</td>
<td>1499</td>
</tr>
</tbody>
</table>

- Solution for standard curve

Pipette 30 μl of RNAc and 1470 μl of H₂O to a 1.5 ml tube to prepare stock solution of 2000 ng/ml. Pipette 650 μl of H₂O the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly and change pipette tips between each transfer. The 1000 ng/ml standard serves as the high standard. H₂O serves as the zero standard (0 pg/ml), shown as Table A2.
4. Procedure

(1) Prepare all reagents, working standards, and samples as directed in the previous sections.
(2) Pipettes 100 µl RNA sample and standard solutions to a 96-well plate, respectively. For each sample, repeat 2 times.
(3) Pipettes 100 µl Ribogreen working solution (1/200) to each well.
(4) Read optical density (OD) in 30 min.

Table A2. Preparation of solution for standard curve.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Concentration (ng/ml)</th>
<th>H₂O (DEPC, µl)</th>
<th>RNAc (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stock</td>
<td>2000</td>
<td>1470</td>
<td>30 RNAc</td>
</tr>
<tr>
<td>S-1</td>
<td>1000</td>
<td>650</td>
<td>650 (Stock)</td>
</tr>
<tr>
<td>S-2</td>
<td>500</td>
<td>650</td>
<td>650 (S-1)</td>
</tr>
<tr>
<td>S-3</td>
<td>250</td>
<td>650</td>
<td>650 (S-2)</td>
</tr>
<tr>
<td>S-4</td>
<td>125</td>
<td>650</td>
<td>650 (S-3)</td>
</tr>
<tr>
<td>S-5</td>
<td>62.5</td>
<td>650</td>
<td>650 (S-4)</td>
</tr>
<tr>
<td>S-6</td>
<td>31.25</td>
<td>650</td>
<td>650 (S-5)</td>
</tr>
<tr>
<td>S-7</td>
<td>15.12</td>
<td>650</td>
<td>650 (S-6)</td>
</tr>
<tr>
<td>Blank</td>
<td>0</td>
<td>650</td>
<td>0</td>
</tr>
</tbody>
</table>

5. Calculation of results

(1) Average the duplicate readings for each standard, control, and sample and subtract the average zero standard value.
(2) Create a standard curve by reducing the data using computer software.
(3) Calculate sample concentration.
6. Example for RNA quantification

Shown as Table A3 and Figure A2.

Table A3. Record value of stand curve for RNA quantification.

<table>
<thead>
<tr>
<th>Solutions</th>
<th>Con. (ng/ml)</th>
<th>Mean (OD)</th>
<th>Cal. Con. (ng/ml)</th>
<th>Residual</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cal1</td>
<td>1000</td>
<td>353</td>
<td>973.21</td>
<td>26.792</td>
</tr>
<tr>
<td>Cal2</td>
<td>500</td>
<td>202</td>
<td>559.23</td>
<td>-59.232</td>
</tr>
<tr>
<td>Cal3</td>
<td>250</td>
<td>88</td>
<td>247.43</td>
<td>2.5718</td>
</tr>
<tr>
<td>Cal4</td>
<td>125</td>
<td>39</td>
<td>115.16</td>
<td>9.8391</td>
</tr>
<tr>
<td>Cal5</td>
<td>62.5</td>
<td>16</td>
<td>51.475</td>
<td>11.025</td>
</tr>
<tr>
<td>Cal6</td>
<td>31.25</td>
<td>6</td>
<td>23.499</td>
<td>7.7512</td>
</tr>
<tr>
<td>Cal7</td>
<td>15.62</td>
<td>3</td>
<td>14.372</td>
<td>1.2531</td>
</tr>
</tbody>
</table>

Formula

\[ Y = 0.365 \times X - 2.583 \]

Corr. Coeff. 0.994

Figure A2. Standard curve for RNA quantification.
APPENDIX IV Protocol of RT-PCR

Reverse transcription (RT)

1. Objective

RNA is reversely transcribed into cDNA using reverse transcriptase.

2. Regents

- M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA, Cat. No. 28025-013)
- Buffer (MMLV-RT) 5X
- Primers, random hexamers (Amersham Pharmacia Biotech, Inc., QC, Canada)
- dNTP DEPC (10 mM)
- Tubes (0.5 ml)
- RNA sample solution (1 µg RNA in 10 µl H₂O)
- MyCycler thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA)

3. Procedures

1. Prepare RNA sample solution (1 µg RNA in 10 µl H₂O).
2. Prepare reaction solutions (Table A4) by adding 2.0 µl H₂O, 4.0 µl buffer (MMLV-RT, 5X), 2.0 µl hexamer (2.5 µg/µl), 1.0 µl dNTP DEPC (10 mM), and 1.0 µl Enzyme (MMLV-RT) (200 U/µl).
3. Heat RNA sample solution at 65°C for 10 min.
4. Add 10 µl reaction solutions to each sample solution, and incubate at 37°C for 60 min.
5. Incubate mixture at 65°C for 10 min. Store solution at 4°C.

Table A4. Preparation of solution for RT.

<table>
<thead>
<tr>
<th>Reaction solutions</th>
<th>Final con.</th>
<th>Volume (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O (DEPC)</td>
<td>---</td>
<td>2.0</td>
</tr>
<tr>
<td>Buffer (MMLV-RT) 5X</td>
<td>1X</td>
<td>4.0</td>
</tr>
<tr>
<td>Hexamer (2.5 µg/µl)</td>
<td>250 ng/µl</td>
<td>2.0</td>
</tr>
<tr>
<td>dNTP DEPC (10 mM)</td>
<td>500 µM</td>
<td>1.0</td>
</tr>
<tr>
<td>Enzyme (MMLV-RT) (200 U/µl)</td>
<td>10 U/µl</td>
<td>1.0</td>
</tr>
</tbody>
</table>
Polymerase chain reaction (PCR)

1. Objective

The cDNA of RNA was amplified with gene-specific primers and enzyme.

2. Regents

- EnzTaq DNA Polymerase (Qiagen, Valencia, CA, USA, Cat. No. 201205)
- GADPH primer
- IL-6 primer
- IL-8 primer
- Buffer (PCR) 10X
- dNTP DEPC (10 mM)
- MgCl₂ (25 mM)
- Tubes (0.5 ml)
- cDNA solution
- MyCycler Thermal Cycler (BIO-RAD)

Table A5. Preparation of solution for PCR.

<table>
<thead>
<tr>
<th>Reaction solutions</th>
<th>Final con.</th>
<th>GADPH</th>
<th>IL-6</th>
<th>IL-8</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O (DEPC)</td>
<td>---</td>
<td>18.62</td>
<td>18.62</td>
<td>18.62</td>
</tr>
<tr>
<td>Buffer (PCR) 10X</td>
<td>1X</td>
<td>2.5</td>
<td>2.5</td>
<td>2.5</td>
</tr>
<tr>
<td>Primer* (50 μM)</td>
<td>2 μM</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>dNTP DEPC (10 mM)</td>
<td>200 μM</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>MgCl₂ (25 mM)</td>
<td>1.25 μM</td>
<td>1.25</td>
<td>1.25</td>
<td>1.25</td>
</tr>
<tr>
<td>EnzTaq Polymerase (5 U/μl)</td>
<td>1.25 U/μl</td>
<td>0.125</td>
<td>0.125</td>
<td>0.125</td>
</tr>
</tbody>
</table>

*Specific primer for GADPH, IL-6 and IL-8, respectively.

3. Procedures

(1) Prepare PCR reaction solutions (Table A5) by adding 18.62 μl H₂O, 2.5 μl buffer (PCR, 10X), 1.0 μl primer (50 μM), 0.5 μl dNTP DEPC (10 mM), 1.25 μl MgCl₂ (25 mM) and 1.0 μl EnzTaq DNA Polymerase (5 U/μl) for each sample.
(2) Mix 1 μl cDNA solution from RT reaction and 24 μl PCR reaction solution.
(3) Run the following program shown as Table A6 using Thermal Cycler.

Table A6. Program of PCR.

<table>
<thead>
<tr>
<th>Step</th>
<th>T (°C)</th>
<th>Duration</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>95</td>
<td>2 min</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>94</td>
<td>1 min</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>54</td>
<td>45 sec</td>
<td>35</td>
</tr>
<tr>
<td>4</td>
<td>72</td>
<td>1 min</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>72</td>
<td>10 min</td>
<td>1</td>
</tr>
</tbody>
</table>

4. Quantification of cDNA of GADPH, IL-6 and IL-8

Same as RNA quantification protocol. One example is shown as Figure A3.

Figure A3. SDS-PAGE of GADPH, IL-6 and IL-8.
APPENDIX V Protocol of cytokine secretion by ELISA

IL-6 quantification

1. Principle

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for IL-6 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any IL-6 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for IL-6 is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, an enhanced luminol/peroxide substrate solution is added to the wells and light is produced in proportion to the amount of IL-6 bound in the initial step. A microplate luminometer is used to measure the intensity of the light emitted. Assay sensitivity is at 0.05 - 0.35 pg/ml.

2. Regants based on Human IL-6 Immunoassay kit (R&D Systems, Minneapolis, MN, USA, Cat. No. Q6000B)

- 96-well Microplate coated with a mouse monoclonal antibody against IL-6
- IL-6 Conjugate (polyclonal antibody against IL-6 conjugated to horseradish peroxidase)
- IL-6 Standard (recombinant human IL-8 in a buffered protein base)
- Assay Diluent RD1-83 (buffered protein base)
- Calibrator Diluent RD6-10 (buffered protein base)
- Wash Buffer Concentrate (10X)
- Glo Reagent A (stabilized enhanced luminol)
- Glo Reagent B (stabilized hydrogen peroxide)
- Plate covers (adhesive strips)
- Tubes (1.5 ml)
- Pipettes and pipette tips
- Model 680 Microplate Reader (Bio-Rad, Hercules, CA, USA)

3. Preparation of working solution

- Wash Buffer (1X)
  100 ml Wash Buffer concentrate (10X) + 900 ml deionized H₂O
- Standard stock solution (30,000 pg/ml)
  IL-6 Standard + 0.5 ml deionized H₂O
- Sample solution
  Remove particulates by centrifugation.
  Mix 50 µl Calibrator Diluent RD6-10 with 50 µl supernatant.
Solution for standard curve
Pipette 950 μl of Calibrator Diluent RD6-10 into the 1500 pg/ml tube. Pipette 800 μl of Calibrator Diluent RD6-10 into the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly and change pipette tips between each transfer. The 1500 pg/ml standard serves as the high standard. Calibrator Diluent RD6-10 serves as the zero standard (0 pg/ml).

4. Procedures

(1) Prepare all reagents, working standards, and samples as directed in the previous sections.
(2) Add 100 μl of Assay Diluent RD1-83 to each well. Each sample was repeated 2 times. Mix well before and during use.
(3) Add 100 μl of Standard or sample per well and incubate for 2 hours at room temperature.
(4) Aspirate each well and wash 4 times.
(5) Add 200 μl of IL-6 Conjugate to each well and incubate for 3 hours at room temperature.
(6) Prepare Working Glo Reagent.
(7) Aspirate each well and wash 4 times.
(8) Add 100 μl of Working Glo Reagent Solution to each well and incubate for 5 - 20 minutes at room temperature on the benchtop. Protect from light.
(9) Determine the RLU of each well using a luminometer set.
(10) Average the duplicate readings for each standard, control, and sample and subtract the average zero standard RLU.
(11) Create a standard curve by reducing the data using computer software capable of generating a cubic-spline or quadratic curve fit.
(12) Calculate sample concentration.
5. Example for IL-6 test by ELISA

Shown as Table A7 and Figure A4.

Table A7. Record value of stand curve for IL-6 test by ELISA.

<table>
<thead>
<tr>
<th>Solutions</th>
<th>Con. (pg/ml)</th>
<th>Mean (OD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stan1</td>
<td>300.00</td>
<td>2.264</td>
</tr>
<tr>
<td>Stan 2</td>
<td>100.00</td>
<td>0.878</td>
</tr>
<tr>
<td>Stan3</td>
<td>50.00</td>
<td>0.4025</td>
</tr>
<tr>
<td>Stan4</td>
<td>25.00</td>
<td>0.1985</td>
</tr>
<tr>
<td>Stan5</td>
<td>12.50</td>
<td>0.1000</td>
</tr>
<tr>
<td>Stan6</td>
<td>6.25</td>
<td>0.0570</td>
</tr>
<tr>
<td>Stan7</td>
<td>3.12</td>
<td>0.0255</td>
</tr>
</tbody>
</table>

Formula

\[ Y = 0.00757 \times X + 0.0236 \]


0.999

Figure A4. Standard curve for IL-6 test by ELISA.
IL-8 quantification

1. Principle

This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for IL-8 has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any IL-8 present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for IL-8 is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, an enhanced luminol/peroxide substrate solution is added to the wells and light is produced in proportion to the amount of IL-8 bound in the initial step. A microplate luminometer is used to measure the intensity of the light emitted. Assay sensitivity is at 0.12 - 0.97 pg/ml.

2. Regents based on Human IL-8 Immunoassay kit (R&D Systems, Minneapolis, MN, USA, Cat. No. Q8000B)

- 96-well Microplate coated with a mouse monoclonal antibody against IL-8
- IL-8 Conjugate (polyclonal antibody against IL-8 conjugated to horseradish peroxidase)
- IL-8 Standard (recombinant human IL-8 in a buffered protein base)
- Assay Diluent RD1-86 (buffered protein base)
- Calibrator Diluent RD6P (animal serum)
- Wash Buffer Concentrate (10X)
- Glo Reagent A (stabilized enhanced luminol)
- Glo Reagent B (stabilized hydrogen peroxide)
- Plate covers (adhesive strips)
- Tubes (1.5 ml)
- Pipettes and pipette tips
- Model 680 Microplate Reader (Bio-Rad, Hercules, CA, USA)

3. Preparation of working solution

- Wash Buffer (1X)
  100 ml Wash Buffer concentrate (10X) + 900 ml deionized H₂O
- Standard stock solution (25,000 pg/ml)
  IL-8 Standard + 0.5 ml deionized H₂O
- Sample solution
  Remove particulates by centrifugation.
  Mix 50 µl Calibrator Diluent RD6P with 50 µl supernatant.
- Solution for standard curve
  Pipette 400 µl of Calibrator Diluent RD6P into each tube. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly and change pipette tips between each transfer. The 5000 pg/ml standard serves as the high standard. Calibrator Diluent RD6-P serves as the zero standard (0 pg/ml).
4. Procedures

(1) Prepare all reagents, working standards, and samples as directed in the previous sections.

(2) Add 100 μL of Assay Diluent RD1-86 to each well. Each sample was repeated 2 times. Mix well before and during use.

(3) Add 50 μL of Standard, sample or control to each well and incubate for 2 hours at room temperature.

(4) Aspirate each well and wash 4 times.

(5) Add 200 μL of IL-8 Conjugate to each well and incubate for 3 hours at room temperature.

(6) Prepare Working Glo Reagent.

(7) Aspirate each well and wash 4 times.

(8) Add 100 μL of Working Glo Reagent Solution to each well and incubate for 5 - 20 minutes at room temperature on the benchtop. Protect from light.

(9) Determine the RLU of each well using a luminometer set.

(10) Average the duplicate readings for each standard, control, and sample and subtract the average zero standard RLU.

(11) Create a standard curve by reducing the data using computer software capable of generating a cubic-spline or quadratic curve fit.

(12) Calculate sample concentration.

5. Example

Same as IL-6 ELISA test.
Publication list


