A reinforced soft polypyrrole membrane and its application in electrically simulated culture of human skin keratinocytes

Thèse

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Une membrane souple à base de polypyrrole renforcée et son utilisation pour délivrer des stimulations électriques aux kératinocytes de peau humaine

Thèse

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Résumé

La stimulation électrique (SE) semble favoriser la cicatrisation des plaies par ses effets sur les fibroblastes. Cependant, son interaction avec les kératinocytes n'a pas été bien établie. Le polypyrrole (PPy) en tant que biomatériau conducteur est un excellent candidat pour délivrer les SE aux cellules, ce qui devient plus évident avec le développement de la nouvelle membrane souple à base de PPy. Cependant, les faibles propriétés mécaniques limitent l'utilisation de cette membrane. La présente étude visait à améliorer la résistance mécanique de la membrane à base de PPy et étudier les comportements cellulaires et moléculaires des kératinocytes après exposition à des SE via cette nouvelle membrane PPy. Premièrement, la membrane souple à base de PPy a été renforcée par électrofilage, de manière synergique, avec des fibres de polyuréthane (PU) et de polylactide (PLLA). Des tests mécaniques ont confirmé que la résistance à la traction de la membrane a été considérablement augmentée. Ensuite, les kératinocytes ont été cultivés sur la membrane PPy renforcée, puis stimulés par des intensités électriques de 100 ou 200 mV mm^{-1} pendant 6 ou 24 heures. Les cellules stimulées présentaient une capacité proliférative considérablement accrue. Les sécrétions d'IL-6, IL-1α, IL-8, GROα, FGF2 et VEGF-A ont également augmenté. Fait intéressant, l'SE de 24 heures a induit une « mémoire de stimulation » car les cellules stimulées ont montré une augmentation significative de formation de colonies (CFE) après 6 jours après l'exposition à la stimulation électrique. De plus, l'expression des kératines 5, 14 et 10/13 était significativement augmentée par la SE. La SE a augmenté l'expression de la phosphorylation des kinases ERK1/2. L'expression des protéines des kératinocytes de la peau humaine peut être activée par des stimulations électriques appropriées pour favoriser la cicatrisation des plaies cutanées. La membrane PPy souple renforcée peut servir de pansement conducteur pour faciliter l'exposition de la plaie à une stimulation électrique pour favoriser sa cicatrisation.

Abstract

Keratinocytes as the principal skin cell type play a major role in wound closure. In the meantime, electrical stimulation (ES) has been found effective in promoting wound healing. However, the role of ES on keratinocytes has not been well established. Polypyrrole (PPy), especially the recently developed soft PPy membrane, is an electrically conductive biomaterial and a good candidate to deliver ES to cells. However, the weak mechanical strength of the soft PPy membrane has limited its practical use. The present work was to enhance the mechanical strength of this soft PPy membrane and to investigate the cellular and molecular behaviors of the keratinocytes underwent ES via this novel PPy membrane. Firstly, the soft PPy membrane was synergically reinforced with polyurethane (PU) and poly (L-lactic acid) (PLLA) fibers through electrospinning technology. Mechanical tests confirmed the significantly increased tensile strength, which rendered the originally fragile PPy membrane strong enough to stand ordinary manipulations without compromising its electrical properties. Afterwards, HaCaT keratinocytes were cultured on the PU/PLLA reinforced PPy membranes under electrical intensities of 100 and 200 mV mm^{-1} for 6 or 24 hr. The electrically stimulated cells exhibited a considerably increased proliferative ability. Meanwhile, secretions of the IL-6, IL-1α, IL-8, GROα, FGF2 and VEGF-A increased as well. Interestingly, the 24 hr ES induced a "stimulus memory" by showing a significant rise in colony forming efficiency (CFE) 6 days post-ES. Additionally, the expressions of keratin 5, keratin 14, keratin 10 and keratin 13 were significantly modulated by ES. Finally, the phosphorylation of ERK1/2 kinases was regulated by ES. The overall results demonstrated that the proliferation, differentiation, and protein expression of human skin keratinocytes can be activated through appropriate ES to benefit skin wound healing. Moreover, the PU/PLLA reinforced soft PPy membrane may server as a conductive wound dressing to facilitate ES to wound.

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Nomenclature

AC	alternating current
AChR	acetylcholine receptor
AKT	Ak strain transforming
AMP	adenosine monophosphate
ATP	adenosine triphosphate
bFGF	basic fibroblast growth factor
BMZ	basement membrane zone
BMP	bone morphogenetic protein
CCL	chemokine (C-C motif) ligand
CFE	colony forming efficiency
CMs	cardiomyocytes
CPs	conductive polymers
CTGF	connective tissue growth factor
CXCL	chemokine (C-X-C motif) ligand
DC	direct current
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
ECM	extracellular matrix
EF	electrical field
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EMT	epithelial-mesenchymal transition
ERKs	extracellular signal-regulated kinases
ES	electrical stimulation
ESCs	epidermal stem cells
F_e	electrostatic force
FGF	fibroblast growth factor
FGF-2 (bFGF)	fibroblast growth factor-2
F_{HD}	hydrodynamic drag force
F_m	mechanical force
FTIR	Fourier transform infrared spectroscopy
GFs	growth factors
GM-CSF	granulocyte-macrophage colony-stimulating factor
GMP	granulocyte-monocyte progenitor
GROα	growth-regulated oncogene α
hBD	human β-defensins
HB-EGF	heparin-binding EGF-like growth factor
HDM2	human double minute 2
HF	hair follicle
HGF	hepatocyte growth factor
HOMO	the highest occupied molecular orbital
IFE	interfollicular epidermis
IFN-γ	interferon γ
IGF-1	insulin-like growth factor-1
IL	interleukin

IP-10	interferon gamma-induced protein 10
IRS	inner root sheet
KGF	keratinocyte growth factor
K	keratin protein
K5	keratin 5
K10	keratin 10
K13	keratin 13
K14	keratin 14
LDH	lactate dehydrogenase
LL-37	a 37 amino acid cationic peptide
LUMO	the lowest unoccupied molecular orbital
MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemoattractant protein-1
MEK	mitogen-activated protein kinase
MMP	matrix metalloproteinase
MO	methyl orange
MRP-1	multidrug resistance-associated protein 1
MS	macrophage stimulating
MTS	methyl thiazolyl tetrazolium salt
MTT	methyl thiazolyl tetrazolium
NaKA	Na^+/K^+-ATPase
NF- κB	nuclear factor-κB
NHE3	Na^+/H^+ exchanger
NMDAR	N-methyl-d-aspartate receptor
ORS	outer root sheet
PA	polyacetylene
PANI	polyaniline
PCL	polycaprolactone
PDGF	platelet-derived growth factor
PEDOT	polyethylenedioxythiophene
PEG	polyethylene glycol
PEMF	pulsed electromagnetic field
PETN	pentaerythritol tetranitrate
PET	poly (ethylene terephthalate)
PGE2	prostaglandin E2
PI3Ks	phosphoinositide 3-kinases
PKB	protein kinase B
PKG	protein kinase G
PKA	protein kinase A
PLA	polylactide
PLGA	poly lactic-co-glycolic acid
PLGF	placental growth factor
PLLA	poly (-L-lactic acid)
PPV	poly (p-phenylene vinylene)
PPy	polypyrrole
PSS	polystyrene sulfonate
PT	polythiophene

PTEN	phosphatidylinositol-3,4,5-trisphosphatase
PU	polyurethane
PVA	poly (vinyl alcohol)
PVDF	polyvinylidene difluoride
Py	pyrrole
p53	tumor protein
Ras	rat sarcoma virus
RHO	Ras homologous
RMP	resting membrane potential
ROS	reactive oxygen species
SB	stratum basale
SC	stratum corneum
SDF	stromal cell-derived factor
SEM	scanning electron microscopy
SG	stratum granulosum
SIVA	apoptosis regulatory protein Siva
SL	stratum lucidum
SOCS	suppressor of cytokine signaling
SS	stratum spinosum
TEP	transepithelial potential
TGA	thermogravimetric analysis
TGF	transforming growth factor
TIMP	tissue inhibitor of metalloproteinases
TLRs	toll-like receptors
TNF-α	tumor necrosis factor α
VEGF	vascular endothelial growth factor
Vm	transmembrane potential
XPS	X-ray photoelectron spectroscopy
α-SMA	alpha smooth muscle actin

TO THOSE WHO DEDICATE THE ENTHUSIASM TO SCIENCE:

Amicus Plato, sed magis amica veritas

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Many thanks to my two supervisors, Dr. Ze Zhang and Dr. Mahmoud Rouabhia, who must have added a lot of gray hairs over the course of these years. At the beginning, I knew almost nothing about research except my enthusiasm. I didn't know how to manage experiment, no focus when reading literature, and my eyes are high, but hands are low. But they were patient and gave me a lot of guidance, which I won't be able to do if I were in their roles. They tolerated my initial lack of knowledge and skills, and they gave me time to grow, which really touched me. Their scientific knowledge, and their sincerity and enthusiasm for science, influenced me and guided me throughout every step of the experiment, every piece of data and paper file, and my whole PhD life. Under their mentorship, I was able to solely focus on exploring scientific questions, and my PhD life became enjoyable, and highly rewarding. When I didn't know how to read literature, they let me choose an article and explained it in the biweekly meetings; when my experimental techniques were unstable, they gave me time to repeat over and over again, to figure out where the problems were; when I didn't know how to write papers, they provided me with valuable advices and revised my manuscripts again and again; they also supported me to attend high-quality academic conferences to keep me up to date with science and technology and to improve my communication ability; when I felt breakdown because of tricky research difficulties, they helped me to clarify the questions and taught me to solve the problems step by step; when I thought I couldn't do, they encouraged me. All of these have inspired me to go forward. And from these, I became able to understand the importance of details; and my mind renewed and became clearer about science and human life. I really don't know how to express my gratitude to them. I am so lucky to have such wonderful supervisors.

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Foreword

This thesis includes two chapters, which come after an introduction regarding the background knowledge related to the research project, followed by general conclusions and perspectives. The research work was conducted in the Centre de Recherche du CHU de Québec located in l'Hôpital Saint-François d'Assise, and in the Oral Ecology Research Group (GREB) in Faculty of Dentistry of Université Laval.

The introduction includes the relationship of bioelectricity and life science, wound healing, and conductive polymers. Also, it describes the motivation, hypothesis, and objectives.

Chapter 1 refers to my first published scientific paper: A biocompatible polypyrrole membrane for biomedical applications. RSC Advances. 2021;11(28):16996-17006. It is authored by Shujun Cui, Jifu Mao, Mahmoud Rouabhia, Saïd Elkoun and Ze Zhang.

Authors' contributions: Conceptualization: ZZ, MR; Funding: ZZ, MR, SE; Investigation: SC, MR, ZZ; Methodology: SC, MR, ZZ, JM; Project administration: ZZ, MR; Original draft: SC. Revision: SC, MR, ZZ, JM, SE. In this paper we demonstrated for the first time the possibility of improving the mechanical properties of the new soft PPy membrane while keeping its conductivity and cytocompatibility.

Chapter 2 refers to my second published scientific paper as follow: Effects of electrical stimulation on human skin keratinocyte growth and the secretion of cytokines and growth factors. Biomedical Materials. 2021;16(6):065021. It is authored by Shujun Cui, Mahmoud Rouabhia, Abdelhabib Semlali1 and Ze Zhang.

Authors' contributions: Conceptualization: ZZ, MR; Funding: ZZ, MR; Investigation: SC, ZZ, MR, AS; Methodology: SC, MR, AS; Project administration: ZZ, M R; Original draft: SC, MR; Revision: ZZ, MR, AS. In this paper we presented innovative results about the interactions of skin keratinocytes with electrical stimulation. We demonstrated that ES could be a valuable tool to promote skin wound healing.

This thesis is concluded with a general discussion, conclusions, and perspectives.

Introduction

I.1 Bioelectricity

"Bioelectricity" is any electrical phenomenon that is actively generated by cells or that is applied to cells to affect cell behaviors [1].

I.1.1 Endogenous electrical phenomenon

All cells are bioelectric, they can use energy to move ions (current) [2-4]. Indeed, multi-scale bioelectric gradients exist in vivo. Besides at cell level (cell membrane potential), electric potential is also detected in organelles (*i.e.*, nuclear envelope), tissues (*i.e.*, skin), and the entire organism (*i.e.*, tadpole) [5-7]. As a cell is the fundamental unit of life, the transmembrane potential (V_m) of a cell plays a fundamental role in physiological networks [7, 8]. The physiological foundation of V_m consists of the electrolyte concentration gradients inside and outside of the plasma membrane, this membrane is composed by the insulating lipid bilayer that segregates the ions across the membrane, and the ion channel proteins across the membrane that use energy to transport ions against concentration gradients, as shown in Figure I.1 [9].

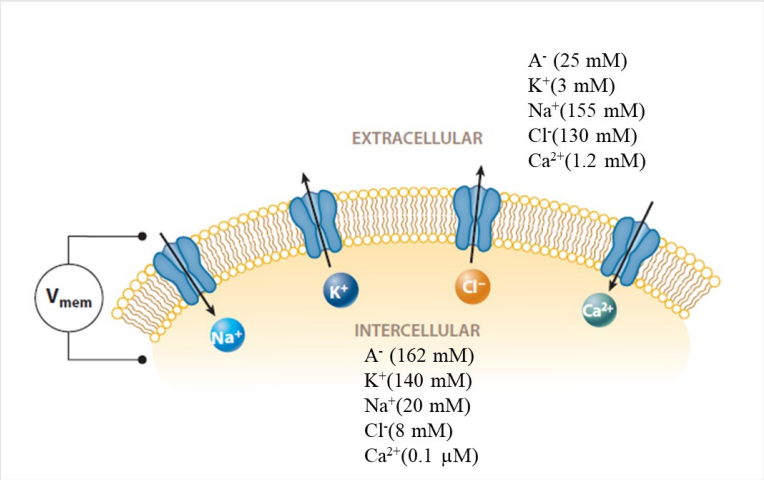

Figure I.1 Transmembrane potential (V_m). Cell maintains V_m through the ion channels and pumps in the membrane [9], and forms an unequal distribution of ions inside and outside of the membrane [10].

The V_m of a single cell is set by the function of the ion channels and pumps in its membrane, and it is not static but changes in time with the cell cycle and long period oscillations [11]. The absolute value of V_m is the sum of all types of ions ($V_{m(ions)}$). At any moment or condition, one type of ion usually dominates, and the V_m is close to the dominated $V_{m(ion)}$, and usually is negative inside [10].

In general, terminally differentiated cells tend to be strongly polarized, *i.e.*, bearing a more negative resting potential, whereas proliferative cells tend to be depolarized, *i.e.*, V_m close to zero. The resting potential is typically cell type specific, depending on cell function and environment [9, 12], as shown in Figure I.2 [13].

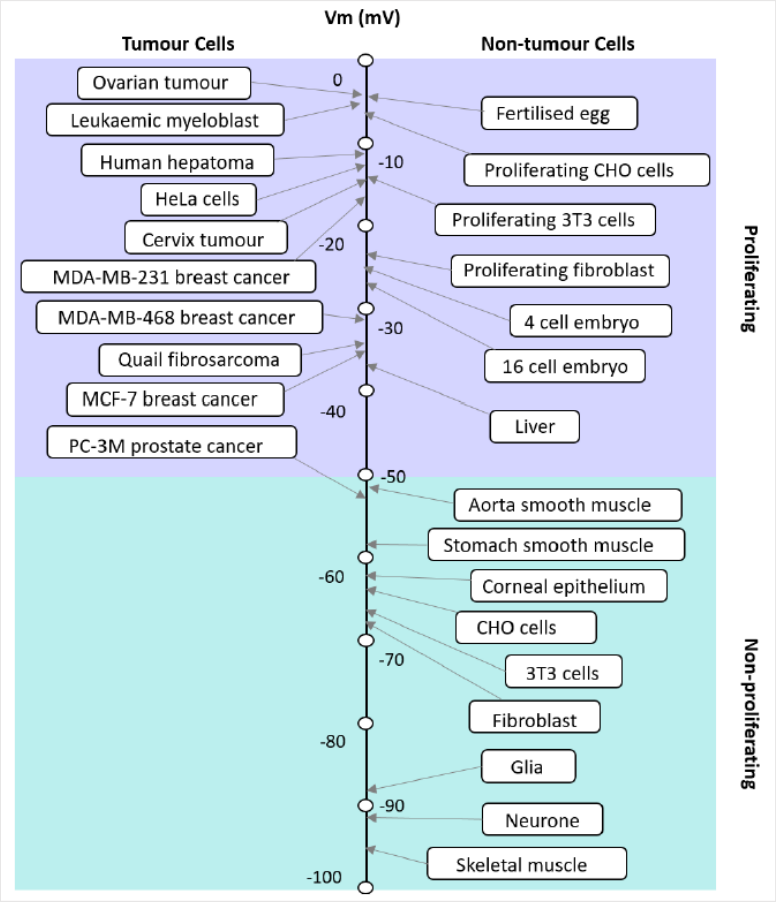

Figure I.2 V_m of different types of cells [13].

To establish bioelectrical networks in large scale, cells exchange electrical signals through membrane proteins with the outside milieu and through gap junctions with the neighboring cells [14, 15]. The bioelectrical signals in form of specific ion currents and changes in V_m can trigger downstream cascades and participate in vast cell activities. For example, they can produce action potential in excitable cells to trigger the well-known biological functions such as the information transfer between neurons, the contraction of skeletal or cardiac muscles, and the insulin releasing by pancreatic β-cells [16, 17]. In fact, this is only a small part of the functions that bioelectrical signals can trigger. The role of bioelectrical signals in non-excitable cells has gained more and more attention. It has been shown that bioelectrical signals are involved in cell proliferation, cell

migration and orientation, cell differentiation, apoptosis, and protein synthesis and secretion [12]. Bioelectrical signals regulate cell cycle, cancer progression, pigmentation, and wound healing [9, 18, 19]. Thus, bioelectric pathways run throughout development, regeneration, and senility, and are crucial for body survival. However, the role of bioelectrical signals is still enigmatic and far beyond our current knowledge [20].

I.1.2 Exogenous electrical stimulation (ES)

Externally supplied current or voltage through biomedical tools can be applied to cells or tissues. This can help us to understand the effect of endogenous electrical signals on cells. It can also provide a method to regulate cell behaviors and eventually lead to new therapies [21].

I.1.2.1 ES methods

In laboratories, several ways have been developed to apply ES. Figure I.3 displays the three main strategies to deliver ES to cells or tissues: 1) In panel (A) the setup is constructed with a petri dish and two electrodes (*i.e.*, graphite, platinum, stainless steel, gold, silver) directly that are immersed in culture medium (Figure I.3A) [22]; 2) ES through a conductive polymer scaffold (Figure I.3B) [23, 24]; and 3) ES through an ionic conductor (Figure I.3C&D) [25].

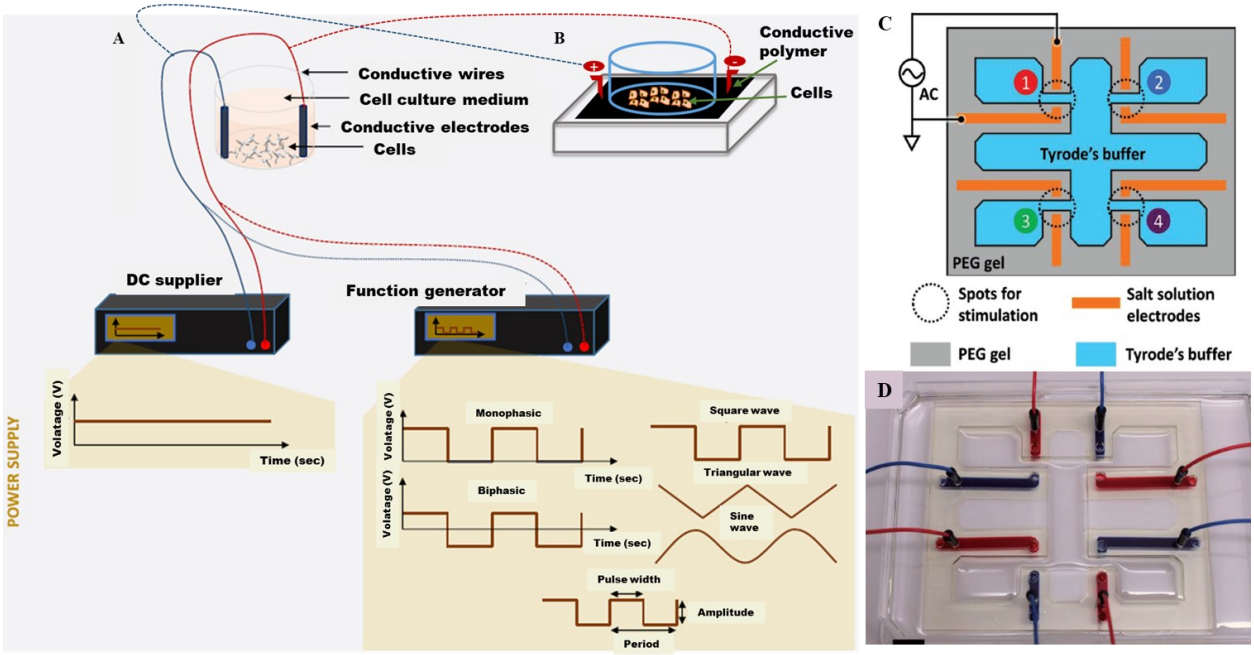

Figure I.3 Electrical stimulation (ES) apparatus for cell culture. A) Cells are seeded in a dish with two electrodes placed in the culture medium [26]; B) Cells are seeded on an intrinsically conductive

polymer scaffold [23]; C) Schematic of the hydrogel ionic conductor for in vitro ES [27]. D) Image of the actual device of C [27]. DC: direct current; AC: alternating current.

An ionic conductor can be prepared with an ionically conductive polymer (polymers with ionic functional groups such as carboxylic, sulfonic, phosphonic, etc.) infused with a high concentration salt solution, *i.e.*, polyethylene glycol (PEG) hydrogel with ionic liquids. Unlike intrinsically conductive polymers whose conductivity relies on electron movement in the polymers, this class of materials conducts electricity in a physiologic environment via the flow of ions upon application of an electrical field (EF) [28]. The advantages of ionic conductors are that they can be made soft and transparent, which is friendly to biological tissues. However, their stability is worrisome in aqueous environment due to the diffusion of ions. Thus, a stable circuit system is needed to interface an ionic conductor with biological tissues [29]. Compared with Figure I.3A, Figure I.3C effectively addressed the issues of adverse effects including local heating and pH change associated with conventional electrodes.

The method showed in Figure I.3B is getting increasingly popular, for the cells grow on the conductive biomaterial directly, which eliminates the adverse effects associated with electrodes such as electrolysis [30]. Intrinsically conductive polymers are organic polymers that conduct electricity through conjugated π-bonds along their molecular backbones. The loosely bound electrons once reduced or oxidized by dopant molecules form charge carriers called polarons and biopolarons that have a high mobility, hence endows the polymer with electrical conductivity [31]. Conductive polymers such as polypyrrole (PPy), polyaceteylene (PA), polyaniline (PANi), polythiophene (PT) and polyethylenedioxythiophene (PEDOT) not only exhibit the good electrical properties, but also display an excellent biocompatibility and the ease of synthesis, which endows them a great potential and advantages in tissue engineering and regenerative medicine [32]. More details about conductive polymers are referred to §I.6.

ES can be induced using a direct (DC) or alternating current (AC) (Figure I.3). The level of the current (A), the amplitude of the voltage (V), and the duration of ES (continuous or cyclic, from hours to days) can be varied. The waveforms (monophasic, biphasic, sine, square, triangle, and others) frequency (Hz) and pulse width (sec) can be altered [26].

I.1.2.2 ES guided cellular responses

When a cell is placed in an EF, the voltage across the plasma membrane will be modified the most

in regions that are perpendicular to the EF lines. The sides of the cells that face the two poles of the field will therefore experience the largest effect [33]. Changes in electrical potential across cell membrane regulate a myriad of effects on the cellular environment such as the distribution of bioactive molecules in extracellular matrix (ECM), as well as have specific effects on membrane proteins and on the enzymes within the cell. These changes may drive cell autonomous phenotypes, such as alignment, migration, proliferation, and differentiation.

The effect of ES on cell alignment and migration can be cell type dependent. Some types of cell are aligned perpendicular to the direction of the EF vectors, such as cardiac adipose tissue-derived progenitor cells, endothelial progenitor cells, adipose-derived stromal cells, etc.; and some cells are aligned parallel to the field vectors, such as ventricular myocytes, cardiomyocytes, myoblasts, etc [21, 34-41]. Previous studies demonstrated that the majority of the cell types investigated so far moves towards the cathode of the EF, such as macrophages, osteoblasts, endothelial progenitor cells, etc.; however, a small proportion of cell types do recorded anodal migration in EF, such as human dermal fibroblasts, Schwann cells, and bone mesenchymal stem cells [35, 42-52].

A proper ES can promote cell proliferation and guide cell differentiation. Osteoblast, neural stem cells and cardiomyocytes showed an enhanced proliferation ability with no morphological changes in EF; on the other hand, the differentiation of human embryonic stem cells, bone mesenchymal stem cells and PC12 were induced under certain ES treatment [21, 53-55].

I.1.2.3 ES guided molecular responses

A current passing through a cell will encounter resistance predominately at cell membrane. Nuccitelli in 2003 speculated that a 100 μm long cell in a 100 mV mm^{-1} EF will experience a voltage drop of 10 mV, with the plasma membrane facing the positive pole having 5 mV more across it, and the membrane facing the negative pole having 5 mV less [33]. For a cell with a membrane potential of -70 mV, the voltage-gated ion channels open following a depolarization of about 10 - 20 mV, and thus would not be affected by a change of 5 mV. However, ion flux through the open channels can be affected and may result in differential distribution of ions within the cell, because the positive ions near the cathode-facing side of cell membrane may experience a larger force to push them into the cell through these open channels than the positive ions near the anode-facing side of the cell membrane. In cells with membrane potentials that are inherently more depolarized, the effects of such EF-caused ion influx may be more significant. Such cells include

mesenchymal stem cells and un-differentiated cells that have a resting membrane potential (RMP) below -30 mV [45].

Although the specific molecule(s) involved in the cell-ES interactions are still to be identified, some hypotheses have been proposed to explain the mechanisms of cellular responses to ES, as summarized in Figure I.4 [29]. In summary, EF can lead to the ***disruption of structural water,*** causing the cells to release a large amount of trapped calcium ions leading to a calcium wave, which directs the mobility of the cells (Figure I.4A). EF can cause a strong ***hydrodynamic dragging force***, which can result in the intracellular transport of biomolecules (Figure I.4B). EF can also ***change the activity of ion channels***, especially the voltage-gated sodium, potassium, and calcium channels, and these changes create the asymmetric ion flows (Figure I.4C). ***Mechanical forces*** can be induced by EF and applied on the tension-sensitive components on cell membrane, affecting membrane functions such as focal adhesion and cadherin adhesion. (Figure I.4D). EF will create polarity along the EF axis by ***redistributing the charged particles on the membrane***, such as the lipid rafts, which further polarizes cell membrane components (Figure I.4E).

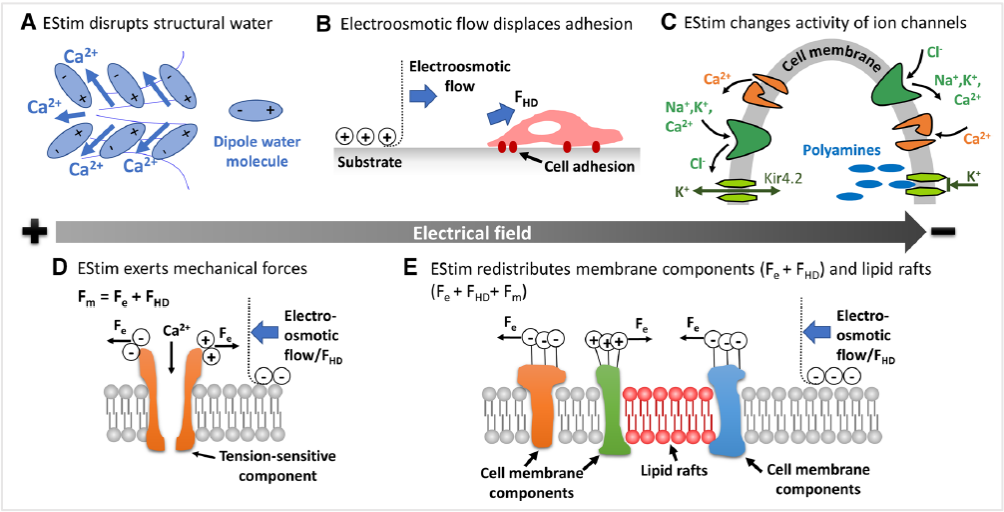

Figure I.4 Models depicting the fundamental physical effects of ES on cells. F_e: electrostatic force, F_{HD}: hydrodynamic drag force, F_m: mechanical forces. EStim: electrical stimulation [29].

The asymmetric localization of cell membrane components and the transport of intracellular biomolecules in response to ES are considered to contribute to the signaling transduction to downstream cascades, and ultimately initiating the cellular activities including migration, proliferation, differentiation, etc [29, 56].

I.1.2.4 ES triggered cell signaling pathways

EF can induce changes in cell membrane components and intracellular biomolecules through F_e, F_{HD} and F_m as mentioned before. However, cellular responses such as orientation, migration and proliferation to EF are not linear. On the way between the aforementioned molecules (Figure I.4) and cell responses, many other messengers inside the cell are engaged to complete certain tasks. In many cases, these messenger cascades can reach the nucleus to activate or inactivate transcription factors, leading to changes in the expression of specific genes. In other cases, messengers will not reach the nucleus and rapid cellular response would occur. In such a way, a cell works like a microprocessor. What needed to be pointed out is that signaling pathways are dynamic and can proceed differently in different cells. And unlike the signaling transduction regulating cellular responses towards chemical gradients, where only the specific surface receptors that bind to chemoattractants are triggers [57], ES can trigger several types of membrane receptors to activate different intracellular signaling cascades. Moreover, the signaling network involving ES shows a coordinated manner. For example, PI3K/Akt pathway can be activated under ES and positively regulate cell electrotaxis. On the other hand, PTEN is only activated where it is away from PI3K in the migrating cells [58, 59]. GTPases Rac/Cdc42/Rho are reported to be able to steer cell migration, during which Rac/Cdc42 dominate cathodal activities, and Rho dominates anodally activities which consequently assist cathodal migration [60]. Therefore, ES can mediate a wide variety of biological processes. The primary signaling network involved in ES-induced cellular response is summarized in Figure I.5 [21, 56, 61].

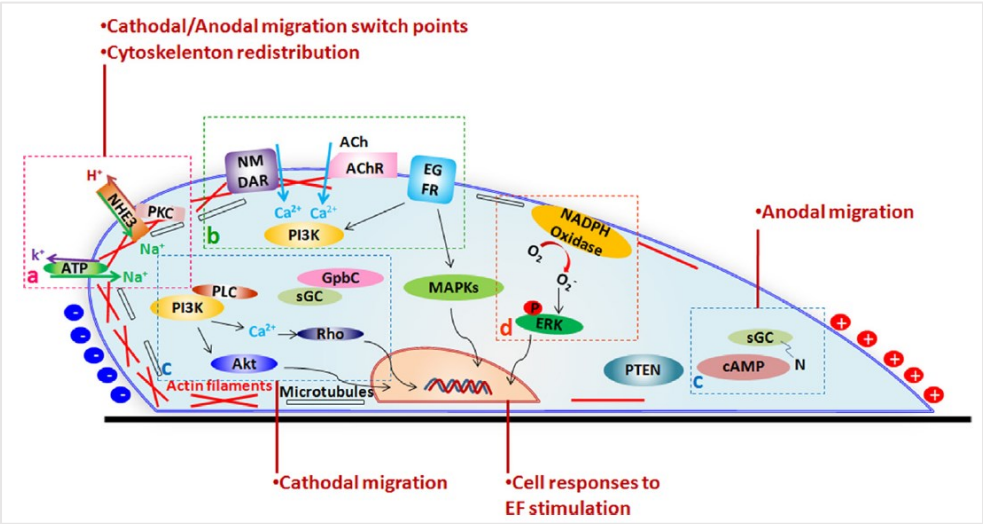

Figure I.5 Signaling network in EF stimulated cell [56].

Signaling pathway related with cell migration. The Na^+ carrier proteins, such as NaKA (Na^+/K^+-ATPase) and NHE3 (Na^+/H^+ exchanger), are sensitive to electrical potential and their distribution in cell membrane becomes polarized upon ES. Inside cells, these proteins interact with actin, tubulin, myosin, etc., and consequently induce cytoskeleton redistribution, leading to membrane polarization and cell migration. AChR (acetylcholine receptor) and NMDAR (N-methyl-d-aspartate receptor) play certain role in inducing Ca^{2+} influxes and cell depolarization. In addition, sGC/cAMP-activated pathway and PTEN (phosphatidylinositol-3,4,5-trisphosphatase) pathway may contribute to EF mediated migration [38, 62-66].

Signaling pathway related with cell proliferation and differentiation. EF-activated EGFR (epidermal growth factor receptor) [67, 68] can stimulate several signaling pathways, including MAPK-ERK1/2 [69, 70] and PI3K/Akt pathways [58, 71]. MAPKs (mitogen-activated protein kinase) is involved in regulating cell functions including proliferation, gene expression, differentiation, survival, and apoptosis. ERKs (extracellular signal-regulated kinases) phosphorylation belongs to MAPKs family and thereby influences the cell responses mentioned above [72]. PI3Ks (phosphoinositide 3-kinases) are a family of enzymes involved in cellular functions such as cell growth, proliferation, differentiation, motility, and survival. The activation of PI3K initiates cellular responses through downstream effector Akt or combining with PLC to activate Rho through Ca^{2+}. Akt, also known as protein kinase B (PKB), plays key roles in multiple cellular processes such as cell proliferation, transcription, and cell migration [21, 73].

I.1.2.5 The potential benefits of ES in medicine and biomedical research

Directing cell alignment and migration, promoting cell proliferation and inducing cell differentiation can be very beneficial for regenerative medicine. Regenerative medicine refers to the replacement of damaged cells or tissues due to injury or disease and remodels the tissue's functional architecture. Therefore, ES can be a useful tool for the purposes of regenerative medicine [74, 75]. Moreover, ES is a non-pharmacological stimulus and can be non-invasive as well. These benefits endow ES in broad applications in medicine and biomedical research.

Neural tissue engineering. ES has been widely used in neural tissue engineering. The effects of ES include the accelerated and directional neurite and axon growth, and the differentiation of embryonic stem cell into the neural fate [76-78].

Muscle tissue engineering. ES has been shown to promote the proliferation of myoblasts, the fusion of myoblasts into myotubes, and the expression of myosin heavy chain [79-81].

Cardiac tissue engineering. ES has been frequently used to facilitate the functional maturation of the stem cell-derived or fetal cardiomyocytes (CMs), including the alignment and elongation of CMs, the increased expression of connexin and troponin-I, as well as the synchronous contractions of CMs within tissue constructs [82-84].

Bone regeneration. ES can stimulate calcium signaling and increase bone formation. ES can also upregulate the production of bone growth factors. When DC-ES is used, the cathode electrochemical reactions generate hydroxide ions and hydrogen peroxide, which have been shown to stimulate osteoblasts and the VEGF production by macrophages. ES in DC, AC, PC and PEMF modes have been tested and shown effective for bone tissue regeneration [85-89].

Drug delivery. At the molecular level, ES can facilitate the transport of both charged and uncharged biomolecules through biological membranes via electrophoresis and electroosmosis. These two processes are collectively called iontophoresis. Compared to passive diffusion, iontophoresis can significantly increase the drug delivery efficiency through tissue barriers such as skin and cornea. Electrophoresis alters the mobility of charged drug molecules through the Coulomb force that the EF exerts on those molecules. Electroosmosis induces a solvent flow across ionized membranes due to the electrical force exerted on the thin electric double layers [90, 91]. In clinic, the EyeGate II delivery system, developed by EyeGate Pharma, uses transscleral iontophoresis to deliver a therapeutic concentration of drug molecules into various ocular tissues [92, 93].

Wound healing. Details are present in §I.4.

I.2 Bioelectricity of skin

I.2.1 General description of the skin

Skin is the largest organ of human body, which acts as a wall to exclude harmful compounds and physical insults, to avoid dehydration and to participate in temperature regulation. Anatomically, the skin is comprised of the epidermis (the outermost layer), the dermis (the next layer which is loaded with blood vessels and nerves), and then the hypodermis [94].

I.2.1.1 The epidermis

The epidermis is a stratified squamous epithelium (as shown in Figure I.6) [95] that creates the chemical and physical barrier between the external environment and the internal body. The thickness of the epidermis can range from 50 μm (eyelids) to 1.5 mm (palms and soles) and it consists of several layers. From the inside to the outside, there are the stratum basale (SB), stratum spinosum (SS), stratum granulosum (SG), stratum lucidum (SL) (palms and soles) and stratum corneum (SC). The SB is anchored to dermis through the basement membrane zone (BMZ), which consists of an intricately organized collection of intracellular, transmembrane, and extracellular proteins [96, 97]. The components of the BMZ include collagen type IV and type VII, laminins, nidogen, and perlecan. There is no direct blood supply in the epidermis. The supply of nutrients and elimination of wastes depends on the underlying dermis by diffusing through the BMZ. Since the BMZ holds the epidermis and dermis together, it plays a critical role in the mutual relationship between keratinocytes and fibroblasts [98].

There are various cell populations including keratinocytes, melanocytes, tactile cells (Merkel cells) and dendritic cells (Langerhans cells) in the epidermis. The predominant cell type is keratinocyte [99]. Melanocytes are located in the SB and they can produce melanin, a pigment that absorbs UV radiation. Merkel cells are also located in the SB and are very close to the nerve endings. These cells are found to specialize in the perception of the light touch. Langerhans cells reside in basal layer but can migrate to get in contact with the antigen. They are the most active immune cells in the skin. Keratinocytes account for 80% of the epidermis' total cellular population and are responsible for the stratified structure of the epidermis. They maintain the constant renewing property of the epidermis, details of which are presented separately [100].

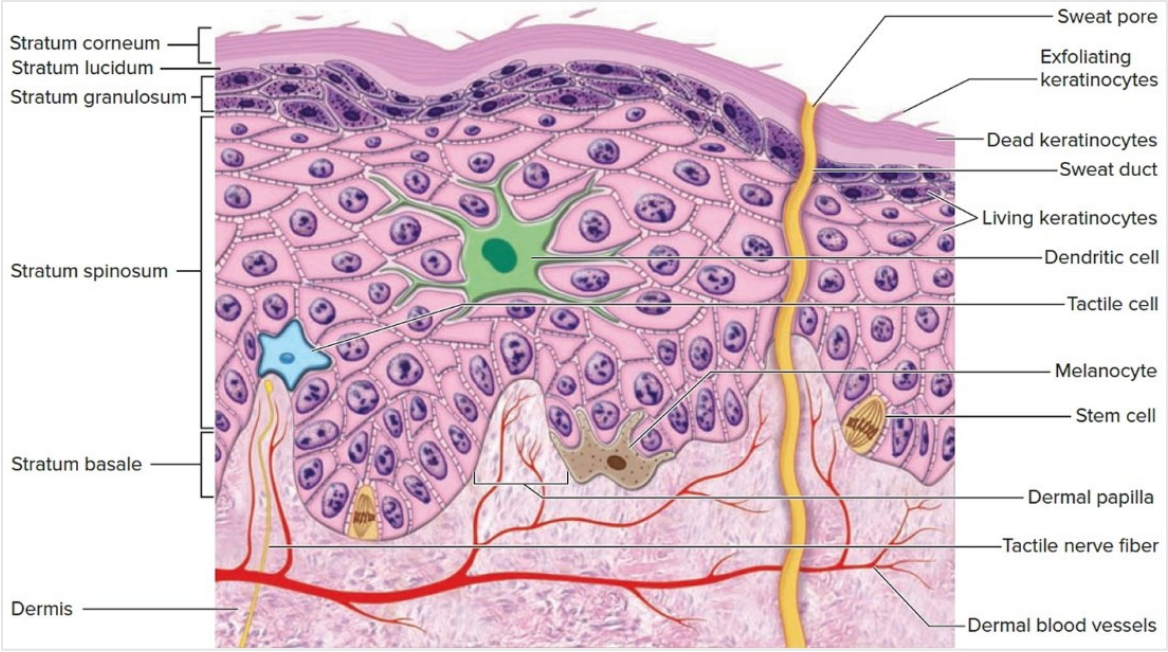

Figure I.6 Structure and skin cells of the epidermis [95].

I.2.1.1.1 Keratinocytes

Keratinocytes define the epidermis through an organized vertical migration accompanied by an equally organized and coordinated sequence of irreversible termination of proliferation and differentiation events, as well as through the formation of the specific connections between cells [100, 101]. The process of epithelialization relies on the special characteristics of keratinocytes and the crosstalk between keratinocytes and other cells; in reverse, these properties endow the epidermis with the functions more than as a physical barrier [102].

I.2.1.1.1.1 Keratinocytes are highly specialized cells in epidermis

The Keratinocytes in different layers of the epidermis are in different stages of differentiation. Their destiny is becoming corneocytes in a process called keratinization, in which they are expulsed of the skin due to the loss of the intercellular desmosomal connections [103, 104]. The basal keratinocyte is highly proliferative and can divide in either symmetric or asymmetric manner (Figure I.7) [105]. Through symmetric division, in which the spindle is oriented parallel to the underlying basement membrane, it produces two daughters in basal layer; and through asymmetric division, in which the spindle is oriented perpendicular to the basement membrane, it gives one basal and one suprabasal keratinocytes [102, 106]. Asymmetric division is coupled with transferring from proliferation to differentiation state, accompanied by synthesizing distinct panel proteins and

soluble mediators and react differentially to exogenous stimuli. This process is highly regulated by several signaling pathways [107, 108].

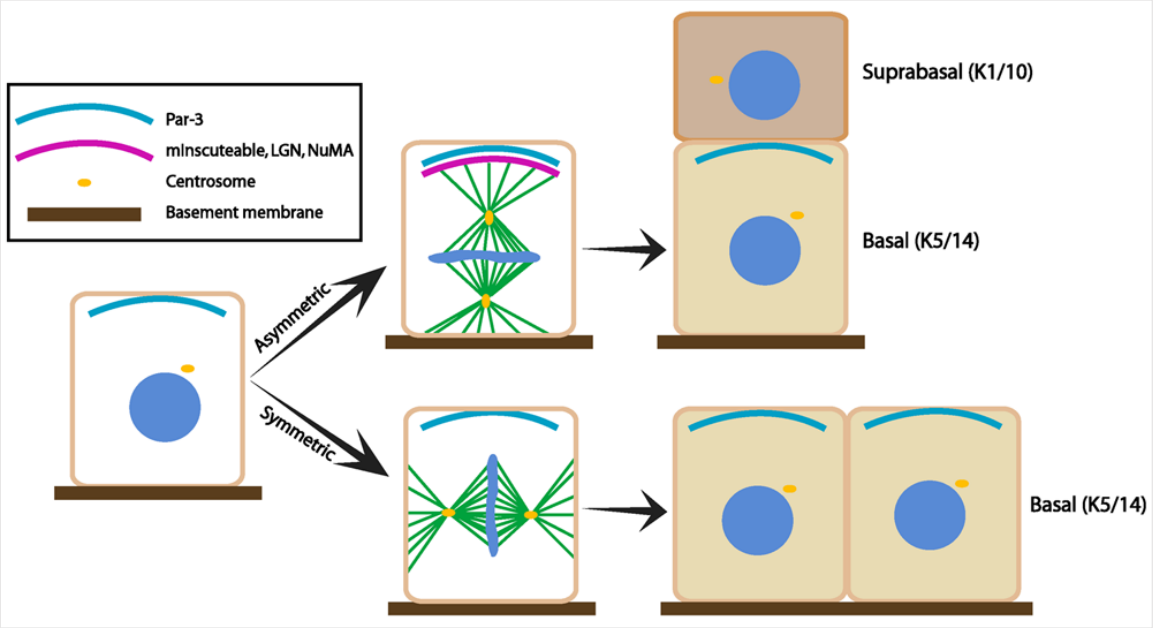

Figure I.7 Model for spindle orientation in symmetric and asymmetric keratinocyte divisions [105].

MAP kinase pathways, which are well-known in regulating this process, can be activated by calcium influx [109], epidermal growth factors [110, 111], and tumor necrosis factors [112, 113]. ERK1/2 promote keratinocyte proliferation [114, 115], while p38 activates the proteolytic and nucleolytic activity that leads to the destruction of cellular organelles and DNA [116, 117]. Notch signaling drives keratin-5/14 positive basal cells into keratin 1/10-positive suprabasal cells [105]. Active EGFR signals promote cell proliferation [118, 119]. Calcium can trigger keratinocyte differentiation via a PKCβ-Calpain-focal adhesion kinase pathway [120]. Following these signaling pathways, once keratinocytes turn profilaggrin into filaggrin, they undergo changes into late terminal differentiation and are irreversibly committed to the process of differentiation [121]. When proteolytic and nucleolytic activities destroy the cellular organelles and DNA, differentiation comes to end [103, 104].

As cells continue their outward journey, they degenerate into a flat crosslinked keratinous material, which forms a cornified envelop on skin surface that is constantly being worn off and replaced by new ones [122]. Lipids and proteins produced by the lamellar granules of keratinocyte in the SG layer fill in the crevices between the corneocytes in the SC layer [123]. It's like a "mortar" and "bricks"

structure which prevents water evaporation from skin [124]. In addition, the stratified layers of the epidermis have developed specific junctions during epithelialization. Indeed, the basal keratinocytes are anchored to BMZ through hemidesmosome [125] and focal adhesion [126]. Adjacent keratinocytes are connected to each other through tight junctions, adherent junctions, and desmosomes. Gap junctions are unique in forming intracellular connection between cell membranes to transfer ionic molecules. These cell-to-cell connections provide the epidermis with a tension-resistant structure capable of supporting shear forces [101, 127].

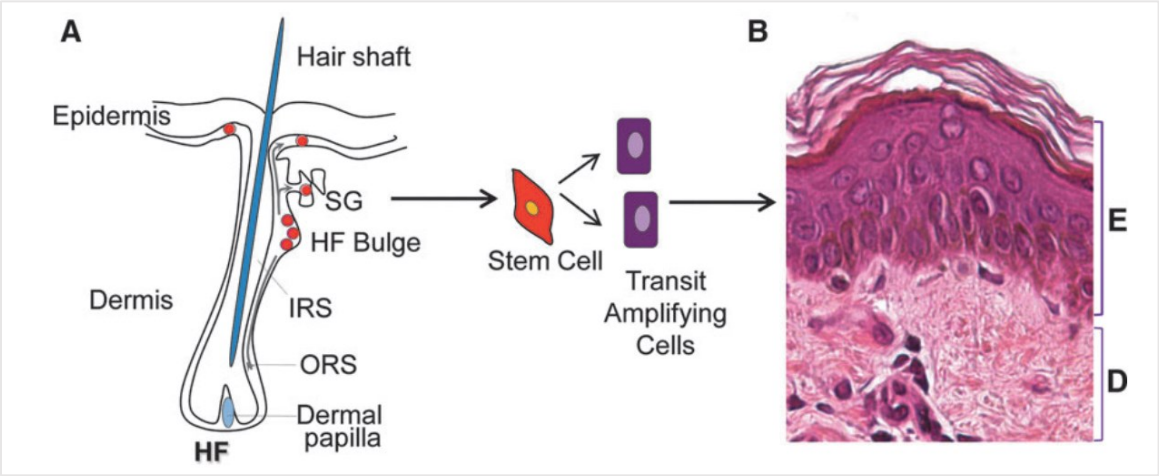

Figure I.8 Regenerative capacity of the epidermis relies on local populations of ESCs. ORS: outer root sheet; IRS: inner root sheet [113].

The renewing capability of keratinocytes is driven by the population of epidermal stem cells (ESC) [102]. There are three distinct ESC niches identified to date (Figure I.8) [113]: the bulge of the hair follicle (HF), the base of sebaceous gland (SG), and the basal layer of the interfollicular epidermis (IFE). The stem cells produce a population of transit-amplifying keratinocytes that have a limited number of division ability. These transit-amplifying keratinocytes either differentiate and depart the basal layer to generate upward differentiating keratinocytes, or remain as proliferative basal keratinocytes [128]. Therefore, damage to these structures affects skin's ability of re-epithelialization.

I.2.1.1.1.2 Polarity in basal keratinocytes

Another important characteristic of keratinocytes is polarity, like the simple monolayer epithelial cells [105, 129-131]. A basal keratinocyte has both basal and apical domains, which are maintained by many polarity components, such as the transmembrane proteins, cell junctions, cytoskeleton, lipids. The polarity is important for generating the proper epidermis architecture. For example,

Par3/Pins/LGN are found in the apical region of the asymmetrically dividing cells, which affect cell fate by directing mitotic spindle orientation [132, 133]; integrin is polarized to the basal side of basal keratinocytes, where it facilitates attachment to the BMZ; primary cilia are enriched at the apical surface of the basal keratinocytes [105]. The primary cilium is an important signaling center, and its apical localization puts it in close contact with the differentiated cells while somewhat away from the signals coming from beneath the cell [134]. Besides these, the polarity has other important roles in maintaining normal life activities, such as skin battery [135], the details of which are presented in §I.2.2.

I.2.1.1.1.3 Cross talk between keratinocytes and other skin cells

The interactions between keratinocytes and other cells are essential for maintaining the skin homeostasis [136-138].

The crosstalk between keratinocytes and fibroblasts has been reviewed by Barbara, et al. in 2020 [136]. Keratinocytes can affect fibroblast activities such as the expression of ECM-related genes or protein production [139], phenotype modification [140], and cytokine production [141]. At the same time, in the presence of fibroblasts, keratinocytes showed enhanced proliferation, reduced apoptosis, physiological differentiation, and enhanced BMZ deposition [142, 143]. In contrast, in the absence of fibroblasts, keratinocytes showed impaired differentiation and dysregulated expression of skin barrier and tight junction proteins, increased skin permeability, and a decreased skin lipid/protein ratio [144]. It has been shown that cytokines such as IL-1 secreted by keratinocytes stimulate fibroblasts to secrete KGF, GM-CSF, TGFα, IL-6, IL-8, IL-1, COX2, and PGE2 [136]. In return, the KGF, GM-CSF and PGE2 secreted by fibroblasts promote keratinocyte proliferation and favor proper keratinocyte differentiation [136]. Keratinocytes contribute to ECM turnover by favoring MMP (Matrix metalloproteinases) over a concomitantly decreased or alternatively increased collagen deposition by fibroblasts [136, 145-147]. However, there are controversial results about the roles of keratinocytes in ECM production by fibroblasts. Thus, more feed-forward mechanisms between these two key cells need to be investigated.

Keratinocyte-melanocyte communication is also crucial for the homeostasis of the epidermis. Melanocytes produce melanin, which absorbs UV radiation and prevents DNA damage to keratinocytes [148]. Keratinocytes, on the other hand, stimulate melanocyte functions such as proliferation, differentiation, and melanogenesis [149, 150].

Keratinocytes have immunomodulatory functions that interact with lymphocytes and Langerhans cells in the skin [137, 138]. Keratinocytes express several Toll-like receptors (TLRs) which can recognize invading microorganisms and initiate host defense reactions [151]. Keratinocytes can produce trigger factors, *i.e.* LL37 cathelecidin/nucleic acid complexes, antimicrobial peptides such as human β-defensins (HBD) and S100 proteins, together with chemokines and cytokines of the IL-1 family that can trigger initial activation of T cells [138, 152-155]. It has been shown that lymphokines released in skin lesions are pivotal for keratinocyte activation and production of inflammatory molecules, which in turn lead to amplification of the local immune responses during wound healing [156, 157].

I.2.1.2 The dermis

The dermis is a fibrous and elastic tissue, with a thickness ranging from 300 μm (eyelid) to 6 mm (soles and palms). It is divided into the superficial papillary layer and the deeper reticular layer. These two layers are separated by a vascular plexus, which is fed by another vascular plexus located at the base of the reticular dermis [94, 96].

The main component of the dermis is ECM, including collagen (70%), elastin (2~4%) and proteoglycans. The collagen provides strength and toughness; elastin fibers maintain normal elastic and flexible features; and proteoglycans provide hydration and viscosity [94, 158, 159]. The papillary dermis is a flowing connective tissue, which is composed of capillaries, elastic fibers, reticular fibers, and non-organized collagen fibers, basically collagen III. The reticular dermis is a compact connective tissue, composed of larger blood vessels, cross-linked elastic fibers, and well-organized fiber bundles of collagen III and I that run parallel to the skin surface [158, 160]. The ECM undergoes constant remodeling, where ECM components are deposited, degraded, or otherwise modified [161].

The most abundant cells in the dermis are fibroblasts, which play a crucial role in the production of ECM and its remodeling. Fibroblasts can release MMPs, which directly regulate ECM production and degradation. In addition, fibroblasts secrete various growth factors (GFs) including the TGF-β and the cytokines such as tumor necrosis factor-alpha (TNF-α) that directly affect keratinocyte proliferation and differentiation. During wound healing, these growth factors and cytokines participate in important processes such as breaking down the fibrin clot, forming the granulation tissue, regulating angiogenesis, supporting wound contraction, assisting re-

epithelization, and creating new ECM [162].

In addition, other cells including dendritic cells and leukocytes are located in, or travel through the dermis. Also skin appendages like sweat and sebaceous glands and hair follicles can be found within the dermis [94].

I.2.1.3 The hypodermis

The hypodermis lies between the dermis and muscle, and its thickness varies from person to person. The main cellular component of the hypodermis is adipocytes structured into lobules separated by the septa that is a fibrous connective tissue containing nerve connections, lymphatic vessels and a rich microvascular network that provides oxygenation and nutrient exchange [94]. The hypodermis provides insulation from the cold, serves as an energy reservoir, protects deep tissues from trauma, and even acts as an endocrine organ by participating in the synthesis of oestrone and leptin [160].

The skin appendages include hair follicles, nails, sebaceous glands, sweat glands, mammary glands, and ceruminous glands. Hair follicles are generated by basal cells in the basement membrane and, apart from palms and soles, they can be found all around the body. They contribute to maintaining body temperature and perceiving touch sensation. Nails are also composed of keratinized and flattened dead cells. Sebaceous glands, located at the base of the hair follicle, secrete an oily substance known as sebum, which lubricates and waterproofs the skin and hair [163]. Sweat glands secrete sweat to the surface of the skin. Mammary and ceruminous glands are modified sweat glands that produce milk and cerumen, respectively [94, 160].

I.2.2 Electrical properties of normal skin

Skin has unique electrical characteristics [2, 164, 165]. The epidermis can be considered a semipermeable membrane, so if there is a difference in ionic strength across this membrane, there will be a potential difference. The dermis and the subcutaneous tissues under epidermis behave in general as pure resistances, which generate negligible DC potentials [11, 166, 167]. This concept is shown in Figure I.9A [135].

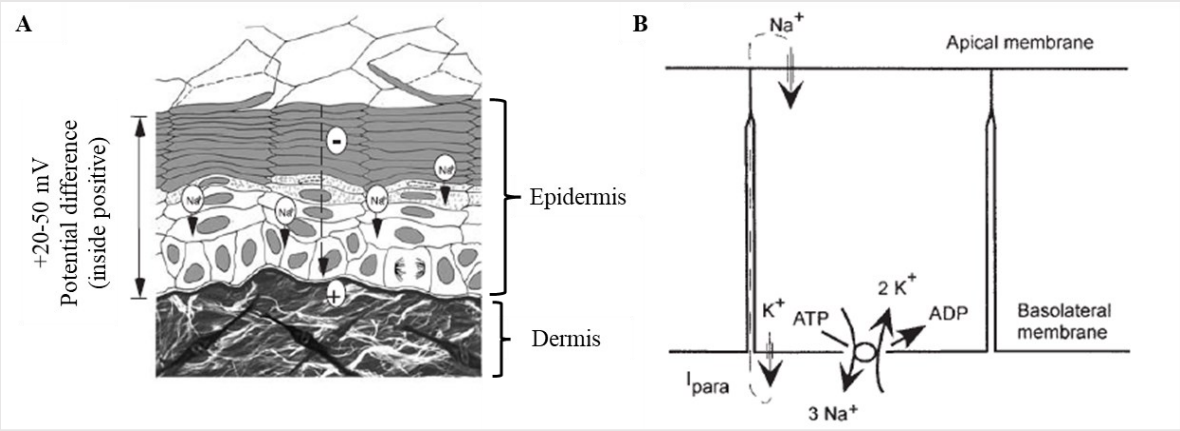

Figure I.9 Generation of electric fields in normal skin. (A) Unbroken skin maintains this ‘‘skin battery’’ or transepidermal potential of 20 - 50 mV. (B) Diagram of a typical epithelial cell in a monolayer with Na^+ and Cl^- channels localized on the apical plasma membrane and K^+ channels localized on the basolateral membranes along with the Na^+/K^+- ATPase [33].

Unlike symmetrical cells, in basal keratinocytes Na^+ channels are localized more in the apical membrane and K^+ channels are localized more in the basolateral membrane. This produces several different transmembrane potentials, among which at least the apical membrane potential and the basolateral membrane potential must be taken into account when it comes to the transcellular potential of keratinocytes [105]. The fact that Na^+ travels across the apical membrane and K^+ travels across the basolateral membrane establish a potential across the cell, with positive charges on the basolateral surface. The apical-basal transcellular current must flow back extracellularly, so the cells prompt to drive Cl^- follow a pathway between the cells, *i.e.*, tight junctions. Because these junctions have high resistance, when ionic current flows through, a transepithelial potential (TEP) is build up, positive to the basal side with respect to the negative apical side, as shown in Figure I.9B [11, 33, 135, 168].

Transcutaneous potential measurements were performed in human body. Negative potentials in the stratum corneum with respect to the dermis were recorded, being -23 ± 9 mV in average [169]. The existence of epidermal “batteries” has been thought to subserve wound healing [170, 171]. Indeed, when decreasing the field strength with the Na^+-channel blocker, wound healing was significantly retarded, while increasing the field strength by the addition of direct current promoted the re-epithelization rate [172-176]. These observations suggest that the endogenous EF plays a role in normal wound healing process.

I.2.3 Electrical properties of wounded skin

When skin is injured, the TEP in wound area becomes zero. The existing TEP in the adjacent intact skin will form a lateral EF to drive the ionic current following the pathway toward the wound center in the deep layer of the epidermis and form an electric circuit around the wound bed (Figure I.10) [33]. As a result, a wound-induced EF is built up and persists until the wound is closed (Figure I.12) [173]. The lateral voltage gradient between the wound center and adjacent intact skin is around 150 mV mm^{-1} and declines with distance from the wound, as shown in Figure I.11 [177]. This lateral voltage gradient falls off gradually over time and ultimately becomes nonexistent due to the increasing resistance created by the newly regenerating epidermis [33, 173, 178].

Cells are also known to respond to EF as a cue to migrate to wound sites. This directional migration, or galvanotropism, due to EF, is thought to mediate early cell migration in wound healing [33]. Studies have shown that almost all skin cells respond to the wound-induced EF. Neutrophils, monocytes, and macrophages show electrotaxis [50, 179], a phenomenon probably beneficial for the immune response and a better antibacterial effect. Endothelial cells respond to EF by projecting the broad, actin-filled lamellipodia [180]. Fibroblasts show time-dependent electrotactic responses [44, 58], not mentioning the electrotaxis of keratinocytes that plays a vital role in wound closure. Inspired by this, scientists have tried to apply ES to accelerate wound closure [181, 182].

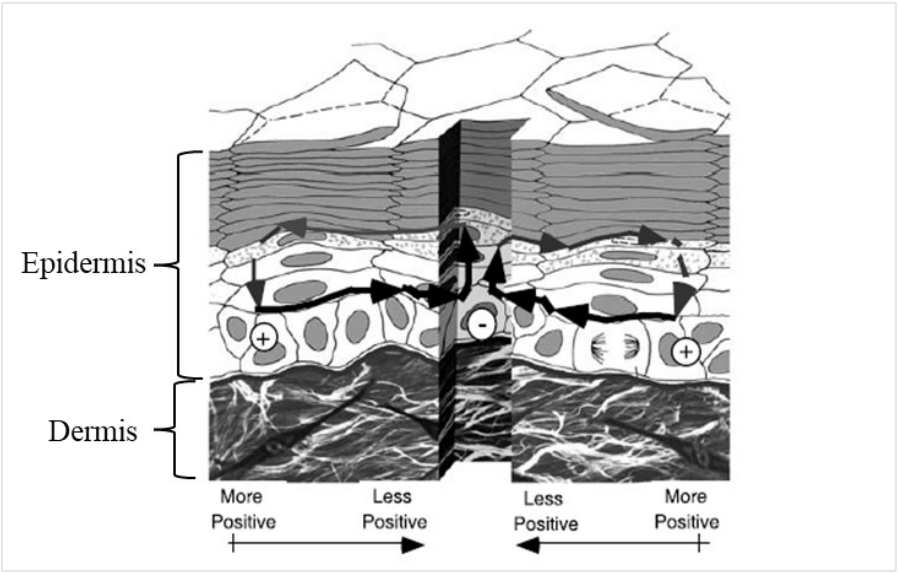

Figure I.10 Generation of electric field in wounded skin. When wounded, the TEP drives current flow through the newly formed low resistance pathway, generating a lateral electric field whose negative vector points toward the wound center at the lower portion of the epidermis and away

from the wound on the upper portion just beneath the stratum corneum [33].

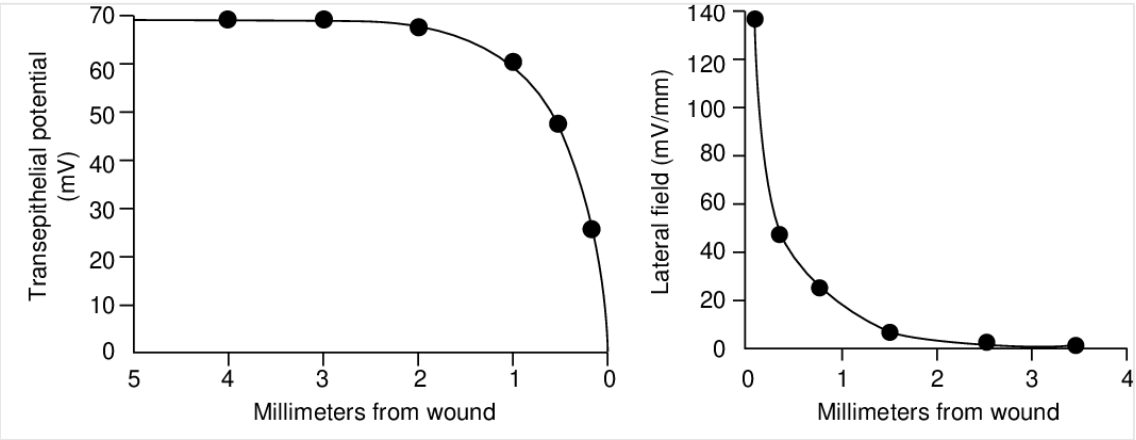

Figure I.11 The magnitude of the natural endogenous electric field near a cut [177].

I.3. Wound healing

Wound healing is an essential biological process that comprises sequential steps aimed at restoring the architecture and function of damaged cells and tissues [183]. Triggered by damage signals, *i.e.*, Ca^{2+} wave, EF, wound healing begins with an immediate gene transcription-independent cellular response, including the changes in cell shape, the formation of functional actomyosin structures and the recruitment of immune cells. This is followed by a gene transcription-dependent response, including cell proliferation, differentiation, and protein production [184]. Overall, wound healing adopts the principle of maximum efficiency to protect the body and restore the tissue function.

I.3.1 Skin wound healing process

As first line of defense, a fast skin wound healing is vital for body survival. It is achieved through several overlapping phases (Figure I.12) [173]: hemostasis, inflammation, proliferation, and remodeling, accompanied with complex signals and regulating factors.

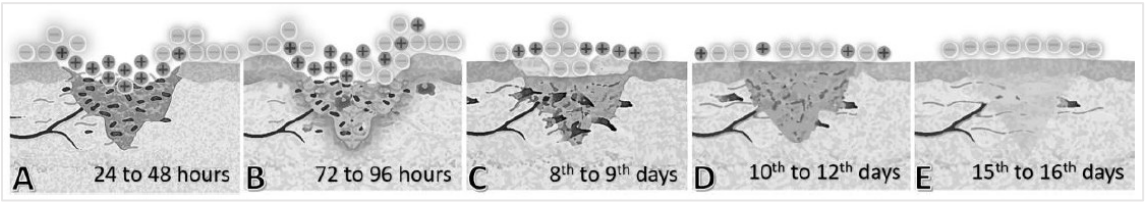

Figure I.12 Skin repair progress [173].

Hemostasis. Immediately after injury, damage signals trigger platelet aggregation at the wound

site. Subsequently, platelet-secreted factors, such as platelet-derived growth factor (PDGF), promote the formation of a fibrin clot to stop bleeding and plugs the wound area to provide a provisional matrix for subsequent cell invasion [185, 186].

Inflammation. While clot formation occurs, leukocytes (neutrophils and later monocytes, which locally differentiate into macrophages) are recruited to the wound. Angiogenesis can be observed at this stage, with the new blood vessels to facilitate the migration of immune cells and the transport of oxygen and nutrients to the wound site. Neutrophils release reactive oxygen species (ROS) and proteases that prevent bacterial contamination and clean cellular debris [186, 187]. At the same time, they release chemokines attracting macrophages and other cells to the wound site. Neutrophils begin to wane when monocytes enter the wound and mature into macrophages. Macrophages phagocytose debris and bacteria, meanwhile secrete extracellular enzymes to degrade necrotic tissue and apoptotic cells, thus paving the way to resolving inflammation [188]. Resolution of the inflammatory phase is accompanied by the apoptosis of inflammatory cells, which are related to anti-inflammatory cytokines and bioactive lipids [189].

Proliferation. This phase is primarily characterized by tissue granulation, newly formed blood vessels, and re-epithelialization. At this stage, growth factors act to induce and maintain cell proliferation and migration, and promote angiogenesis to provide a favorable microenvironment for keratinocytes and fibroblasts [162, 189]. Granulation tissue is a mixture of loosely organized ECM (collagen III, fibrin, fibronectin, hyaluronic acid, etc.) and several cell populations, *i.e.*, fibroblasts, immune cells, and endothelial cells. Mechanical tensions experienced by fibroblasts in the granulation tissue, together with the factors such as PDGF and TGF-α, trigger fibroblast differentiation into myofibroblasts. Myofibroblasts are rich in alpha smooth muscle actin (α-SMA) and so have a high contractile capacity, which causes wound contraction and promotes the wound re-epithelialization [190]. The details of re-epithelialization will be present in §I.3.4.

Remodeling. As the edges of a wound come together, cell migration and proliferation terminate. The immune response also ends. The vasculature is reorganized. MMPs and tissue inhibitors of metalloproteinases allow the replacement of collagen type III into collagen type I, which has a much higher tensile strength, to form a mature scar tissue [191, 192]. Although this scar tissue is enough as a barrier, but it has 30% less mechanical strength than the healthy tissue and lacks skin appendages. A scar tissue never regains the properties of the uninjured skin [193].

I.3.2 Wound healing limitations

Healing can follow two different mechanisms: regeneration or repair. Regeneration achieves a full healing of the original tissue; repair develops into a scar [194]. Human fetuses can regenerate the wound without scarring, but this ability is lost during adult life. A leading hypothesis is that the immune system is involved in the switch between regeneration and fibrotic healing, as human fetuses have immature immune systems [195, 196]. Because the immune response not only combats infections but also can result in the local over-deposition of fibrin fibers and collagen, causing fibrosis and scarring [197]. Another explanation is that a rapid scarring tissue confers a survival advantage by preventing infectious microorganisms from invading the wound and by inhibiting the continued mechanical deformation of larger tissues [183]. However, these mechanisms are not fully understood till now. Thus, a full understand of wound healing, and a better control of inflammation during wound healing is a way to eliminate undesirable side effects and advance to tissue regeneration.

I.3.3 Wound healing regulators

Cells are sensitive to physical and chemical changes. Injured cells release a cocktail of ions and factors which can be detected by adjacent healthy cells. In response to the changes, they send complex damage messages aimed at informing target cells to start wound healing. Subsequently, genes can be turned on or off depending on the distance to the wound and the stage of wound healing [184, 198]. Therefore, there exist both immediate regulators and more sustained signals during wound healing. Hence, understanding these regulators will contribute to develop a better wound healing therapy.

I.3.3.1 Wound signals

Since gene expression takes time, cells must turn-on the readily available mechanisms in order to respond to immediate challenges posed by the wound. To date, the main immediate transcription-independent and diffusible damage signals include Ca^{2+} wave, ROS, purinergic molecules, and EF. Healthy cells can recognize these damage signals and trigger complex repair programs [184].

Ca^{2+} wave. Injury leads to a rapid increase in intracellular Ca^{2+}, which can activate RHO GTPases and promote actin polymerization and actomyosin contractility, possibly through RHO GEFs [199-202]. Furthermore, Ca^{2+} can directly activate actin-severing proteins, such as calpain and gelsolin,

leading to increased actin dynamics. Subsequently, Ca^{2+} can potentiate JNK and ERK signaling, which leads to the transcription factor activation and the increased expression of wound response genes, including several cytoskeletal regulators [203, 204].

ROS. ROS such as H_2O_2 are highly reactive oxygen-containing molecules that are formed in cells as the products of the oxidative metabolism or the activity of oxidative enzymes. Studies have shown that as early as 3 minutes after injury, wound bordering cells experience a rise in H_2O_2 levels; and dissipation of H_2O_2 by one hour after wounding coincides with the decreased immune cell migration to the wound, underlining the predominant role of H_2O_2 as an immediate pro-inflammatory factor [205].

Purinergic signal. Under normal conditions, the intracellular concentration of ATP is very high (approximately 100 mM), whereas the extracellular concentration is considerably lower (approximately 10 nM). Upon injury, a considerable amount of ATP is released by damaged cells into the extracellular milieu. Research shows that the active ATP release and the purinergic receptor-mediated ATP recognition might have an important role in alerting the immune system [206, 207].

Electric field. Damage-induced membrane disruption alters ion transport profiles across cell membranes [165, 169]. This results in the formation of EFs that have essential roles during wound healing [208]. Studies have shown that skin cells display wound-induced electrotaxis [33, 42]. Details will be presented in §I.4.

In addition, these damage signals are integrated and co-regulated. For instance, the intracellular Ca^{2+} wave can promote ATP release, while the consequent engagement of purinergic receptors of neighboring cells leads to a concurrent Ca^{2+} uptake. The Ca^{2+} release by internal stores is also promoted, propagating the initial damage signal. Moreover, ATP and Ca^{2+} cooperatively promote EGFR signaling and activate its downstream PI3K–AKT and ERK–MAPK signaling pathways in epithelial wound healing [184].

I.3.3.2 Cytokines, growth factors and chemokines in wound healing

Damage to cells triggers the activation of conserved signal transduction pathways that ultimately cause changes in the transcription of specific genes. For example, a strong expression of suppressor of cytokine signalling-1 (SOCS-1) mRNA gene will down regulate the proinflammatory cytokines,

while a highly expressed macrophage stimulating-1 (MS-1) gene is a proinflammatory mediator. These two genes balance the inflammation process during wound healing [209]. Cytokines, growths factors and chemokines mediate all these events and regulate cell activities from the injury to the final healed tissue. These agents can bind to specific cell membrane receptors or ECM proteins to trigger a cascade of molecular events. The endpoint of a signaling pathway is the gene promoters that regulate the translation of the proteins that control cell cycle, migration, infiltration, or differentiation patterns [162, 183, 184, 210]. These transcription-dependent cellular responses are slower but more specific. Working in this manner, these factors ensure the success of wound healing. The important factors are summarized in Table I.1.

Table I.1 Important growth factors, cytokines, and chemokines in skin wound healing

Ligand	Receptor location	Secreted by	Function in wound healing	Ref.
EGF	Throughout epidermis, prominent in basal layer	Platelets, macrophages, fibroblasts.	• Accelerate keratinocyte migration and proliferation (increase K6, K16). • Increase fibroblast collagenase secretion. • Inhibits fetal wound contraction.	162, 211-213
TGF-α	Epidermis	Platelets, keratinocytes, macrophages, lymphocytes, fibroblasts.	Accelerate keratinocyte migration and proliferation (through the increase K6, K16).	162, 211, 212, 214, 215
HB-EGF	Epidermis	Keratinocytes	• Promote keratinocyte migration. • Angiogenesis.	162, 212, 216
FGF-2 (bFGF)	Epidermis & dermis	Macrophages, endothelium, keratinocytes, mast cells, fibroblasts.	• Increase keratinocyte motility. • Regulate ECM synthesis and deposition. • Promote fibroblasts proliferation and stimulates collagenase production (collagen remodeling). • Prevent wound contraction in fetal wounds. • Involved in endothelial cell growth and migration, promote angiogenesis.	162, 211
FGF-7 & FGF-10	FGFR2IIIb receptor, solely on epithelium of ectodermal origin	Endothelium fibroblasts. γ-δ T cells.	• Regulate keratinocyte proliferation and maturation. • Detoxification of ROS.	162, 211, 217

FGF-22	FGFR2IIIb receptor	Hair follicle keratinocytes, keratinocytes of hypertrophic epidermis of the wound bed in the late stages of wound healing.	More studies needed to better understand	[162, 212, 216]
TGF-β1/ β2	Epidermis & dermis	Platelets, macrophages, fibroblasts, keratinocytes.	• Recruit inflammatory cells and augment macrophage mediated tissue debridement. • Deactivate superoxide production from macrophages. • Shift keratinocyte integrin expression toward a more migratory phenotype. • Increase the expression of genes associated with ECM formation. • Up-regulate VEGF. • Facilitate contraction. • Inhibit MMPs and promote TIMP synthesis. • Induce arginase activity in several cell lines. • Overexpression can lead to elevated scarring. • Inhibit the same processes that it activates in immune cells. • Fetal wounds lack of TGF-β (scarless healing).	[162, 211, 218, 219]
TGF-β3	Epidermis & dermis	Keratinocytes	• Recruit inflammatory cells and fibroblasts to the wound site and facilitate keratinocyte migration. • Inhibit scarring and promotes better collagen organization. • Stop signal for terminal differentiation in epidermis.	[162, 211, 218, 219]
Activins	Fibroblasts, keratinocytes	Fibroblasts, keratinocytes.	• Affect keratinocyte proliferation indirectly by inducing the expression of growth factors in dermal fibroblasts. • Inhibit keratinocyte proliferation and induce terminal differentiation.	[162, 211]
BMP-6	Keratinocytes	Regenerated keratinocytes, fibroblasts.	• Important in keratinocyte differentiation. • An overexpression has been shown to severely delay re-epithelialization.	[162, 211]
PDGFs	/	Platelets, macrophages, vascular endothelium, fibroblasts, keratinocytes.	• Mitogenicity and chemotaxis of neutrophils, macrophages, fibroblasts to wound site. • Stimulate macrophage-derived growth factors, *i.e.*, TGF-β. • Recruit pericytes to the capillaries.	[162, 211, 220]

			• Increase keratinocyte motility by up-regulating IGF-1. • Enhance fibroblasts proliferation. • Stimulate fibroblasts switch to myofibroblasts. • Up-regulating MMPs to promote tissue remodeling.	
VEGF-A	Endothelial surface of blood vessels	Endothelium, keratinocytes, fibroblasts, macrophages, platelets, neutrophils.	• Promote early angiogenesis. • Play a role in lymph angiogenesis.	162, 220-222
VEGF-C	Lymphatic endothelium, fenestrated endothelia, monocytes, macrophages	Macrophages	• Recruit inflammatory cell to wound site. • Play a role in lymph angiogenesis. • Play a role in angiogenesis.	162, 221, 222
PLGF	/	Keratinocytes, endothelium.	• Promote monocyte chemotaxis and bone marrow-derived precursor cell mobilization. • Promote granulation tissue formation, maturation, and vascularization.	162, 221, 222
CTGF	/	Fibroblasts, muscle cells, γδ-T lymphocytes.	• Stimulate proliferation and chemotaxis of fibroblasts. • Essential for re-epithelialization by promoting keratinocytes migration induced by TGF-β through Ras/MEK/ERK MAPK. • Strong inducer of ECM proteins. • Mesenchymal cell adhesion, migration, and proliferation.	162, 211, 223
GM-CSF	/	Keratinocytes, fibroblasts, endothelial cells, macrophages	• Promote inflammation. • Increase keratinocyte proliferation. • Increase migration and proliferation of endothelial cells.	162, 211
IL-1 (IL-1α, IL-1β)	/	Neutrophils, monocytes, macrophages, keratinocytes.	• Activate neutrophils and promote chemotaxis. • Induce proinflammatory cytokine expression. • Induce keratinocyte migration (expression K6, K16) and proliferation. • Activate fibroblasts and increase FGF-7 secretion. • Elevated levels of IL-1β	162, 211, 224
IL-18	T Lymphocytes, NK cells, macrophages	Keratinocytes, macrophages, dendritic cells.	• Up-regulate Th1 cytokines. • Overproduction induces severe inflammatory disorders.	162, 225, 226
IL-6	/	Neutrophils, monocytes,	• Stimulate keratinocytes and fibroblasts proliferation.	162

		fibroblasts.	• Chemo attractive to neutrophils. • Primary inducer of the hepatic and myeloid acute phase responses. • Inappropriate IL-6 expression is unfavorable to healing.	
TNF-α	/	Macrophages	• Essential in the early phases; overproduction or prolonged expression in remodeling phase causes increased tissue destruction by the over activation of immune cells and their protease products. • Induce neutrophil recruitment and maturation. • Increase hemostasis. • Indirectly promote re-epithelialization by inducing FGF-7 production. • Alone, at low levels, can stimulate inflammation and increase macrophage produced growth factors.	162, 211, 224, 227, 228
IFN-γ	/	T lymphocytes	• Polymorphonuclear leukocytes and macrophage activation and cytotoxicity. • Induce tissue remodeling. • Overexpression can reduce re-epithelialization.	162, 229
IL-2	/	T lymphocytes	• Predominantly a T-cell growth factor. • Associated with inflammation.	162
IL-4	/	T lymphocytes, basophils, mast cells	• Suppress proinflammatory cytokine expression. • Promote fibroblast proliferation. • Up-regulate arginase activity in fibroblasts, macrophages, endothelium.	162
IL-10	/	T lymphocytes, dendritic cells, macrophages	• Inhibit proinflammatory cytokine production. • Prevent neutrophil and macrophage infiltration into the wound.	162
MCP-1 or CCL2	/	Keratinocytes	• Chemoattractant for monocytes/macrophages, T-cells, and mast cells.	210, 230
IP-10 or CXCL10	/	Keratinocytes	• Promote inflammatory response. • Delay re-epithelialization and prolong granulation phase.	210, 230
IL-8 or CXCL8	/	Macrophages, fibroblasts.	• Increase keratinocyte migration, proliferation, maturation, and margination. • Induce MMPs expression in leukocytes, stimulate tissue remodeling. • Strong chemoattractant for neutrophils. • Increase endothelium adhesion protein expression. • Excessive expression causes increased scarring.	210, 230

			• Very low concentrations in fetal tissue.	
GRO-α or CXCL1	/	Keratinocytes	• Promote keratinocyte migration. • Potent regulator of neutrophil chemotaxis.	210, 230
SDF-1 or CXCL12	/	Endothelium, myofibroblasts, keratinocytes.	• Recruit lymphocytes to the wound. • Promote proliferation and migration of endothelial cells. • Recruit bone marrow progenitors from circulation to peripheral tissues. • Enhance keratinocyte proliferation.	210, 230

I.3.4 Re-epithelialization

Re-epithelialization can be viewed as the result of three overlapping keratinocyte activities: migration, proliferation, and differentiation. The sequence of the events begins with the dissolution of cell-cell and cell-substratum contacts, followed by the polarization and initiation of migration in basal, and a subset of suprabasal keratinocytes over the provisional wound matrix. A subset of keratinocytes immediately adjacent to, but not within the wound bed, then undergo mitosis. Finally, keratinocytes stratify and form the new epidermis [231-233].

I.3.4.1 Re-epithelialization mechanism

Re-epithelialization is stimulated by the local wound milieu [234]. Several re-epithelialization mechanisms have been postulated, including “collective leading-edge keratinocytes migration”, “increased progenitor keratinocytes proliferation behind the leading-edge zone” and “partial epithelial-mesenchymal transition (EMT)”. And these mechanisms involve multiple signaling cascades [100, 113, 235-238].

Two distinct keratinocyte zones play critical roles: A proliferative hub is composed of the IFE and HF-derived stem cells and their progeny, and a migrate leading edge composed of the non-proliferative keratinocytes. The migrate leading edge acts as a scaffold secreting a high level of enzymes that remodel the surrounding ECM and fibrin clot, allowing epidermal regeneration to progress toward the center of the wound and protecting the stem cells and their progeny from the immediate vicinity of the wound front and infection [100, 235, 239].

The adhesive complexes of migrating keratinocytes must be stable enough to maintain contact points, yet plastic enough to form the leading edge and disassemble at the trailing edge [240-243]. Consequently, the regulation of junctional dissolution must be very finely tuned to enable just

sufficient loosening between keratinocytes to allow a migration without detaching the migrating cells entirely from one another so they cannot migrate collectively [241, 244, 245], which is the so called "partial EMT". Migration relies on the coordination of forward protrusion and rearward retraction forces, mediated by actin and microtubule cytoskeleton. Reorganization of keratinocyte cytoskeleton lets the keratinocytes in the leading edge acquire a motile phenotype and start migrating [235]. The migrating cells do not proliferate actively as it migrates to the wound center. Proliferation resumes when the edges fuse at midline of the wound.

There are multiple regulatory cascades that modulate re-epithelialization. So far what have been intensively studied include the altered integrin, the laminin-332 expression and distribution associated with migrating keratinocytes, and the hyperproliferative keratinocytes expressing K6, K16 and K17. IGF-1 stimulates membrane protrusion and facilitates cell spreading through PI3K signaling pathway, while EGF has been shown to induce keratinocyte size reduction through MAPK signaling pathway [234, 246-252].

Of the keratinocytes in the leading-edge migrating zone, the most upregulated genes are the genes coding for the proteins that include MMP9, MMP13 and MMP1, whereas TIMP3 is the most downregulated gene, suggesting that a high level of MMPs promotes the remodeling of the ECM at the wound front allowing the front cells to progress toward the center [253]. MMPs also help the breakdown of the hemidesmosomes that anchor cells at basal membrane and are therefore essential for the movement of basal cells. The migrating zone also expresses a high level of urokinase (Plau) and urokinase receptor (Plaur), two key fibrinolytic proteins contributing to the remodeling of blood clot [249, 254-256]. In addition, the genes controlling keratinocyte adhesion, including proto-cadherins (Pcdh7, Pcdhb19, Pcdhga1), integrins (Itga5, Itga6) and their ECM ligands (Fn1, Lama3, Lamb3, Lamc2), desmosomes (Cdsn) and gap junction proteins (Gjb6/Cx30 and Gjb2/Cx26), are alternatively expressed [235]. The genes controlling keratinocyte cytoskeleton remodeling including actin regulators (Fscn1, Cald1, Nav2, Fmnl2), myosin (Myo1b, Myo5b, Myh9, Tpm1, Tpm2) and tubulin (Tubb2a, Tubb3, Tubb6), are preferentially overexpressed. These genes control the morphology, polarity, and migration of keratinocytes. Several genes that regulate cell quiescence (E2f7, Fgf18) may contribute to the shutdown of proliferation [234, 235].

I.3.4.2 Re-epithelialization regulators

Re-epithelialization relies on numerous signaling cues and multiple cellular processes that take place both within the epidermis and in other participating tissues [234, 257]. A variety of modulators are involved (Table I.2).

Table I.2 Important regulators during re-epithelialization [63, 141, 183, 234, 258, 259].

Factors	Functions in re-epithelialization
EGF, HB-EGF, TGF-α	Promote keratinocyte migration and proliferation.
FGF2, FGF7, FGF10	Increase keratinocyte migration, proliferation, and survival.
TGF-β	Contradictory findings: Inhibition/promotion of keratinocyte migration and proliferation.
BMP-6	Inhibit proliferation and induce differentiation of keratinocytes.
HGF	Stimulation of keratinocyte migration and probably proliferation.
GM-CSF, IGF, IFN-γ, IL-6	Increase re-epithelialization.
IL-1	Increase keratinocyte survival, motility, and proliferation.
TNF-α	Indirectly augment re-epithelialization though the expression of FGF-7 while directly inhibit re-epithelialization through keratinocyte p55 receptor. The direct effects seem to play a more dominant role.
IL-6	Regulate keratinocyte motility and wound re-epithelialization.
Chemokines (IL-8 or CXCL8, GRO-α or CXCL1, MCP-1 or CCL2, MRP-1 or CCL6)	Alter keratinocyte migration and/or wound re-epithelialization.
Oxygen	Hypoxemic (2%) can suppress keratinocyte differentiation, increase keratinocyte motility.
Antimicrobial peptides (LL-37)	Increase keratinocyte motility.
ECM	Composition of the underlying ECM can modify the speed of keratinocyte migration in the presence of growth factors.
Epithelial–mesenchymal interactions	Fibroblast-keratinocyte interactions via cytokines and their receptors and signal transduction pathways regulate keratinocyte differentiation and proliferation.
MMPs, TIMPs	MMP and TIMP levels are under tight regulation for optimal keratinocyte motility.
Acetylcholine	M3 receptor, calcium-dependent guanylyl cyclase, cyclic GMP and PKG, leading to the inhibition of RHO and keratinocyte migration. M4 receptor, adenylyl cyclase, cyclic AMP and PKA, leading to the activation of RHO and stimulation of keratinocyte migration.
Catecholamines, including adrenaline	Inhibition of keratinocyte migration
Polyunsaturated fatty acids	Stimulation of keratinocyte migration and survival.

Electric field	EGFR, α6β4-integrin, phosphatidylinositol-3-OH kinase-γ and the phosphatase PTEN, directional cue for the migration of keratinocytes.

I.4 The benefits of ES in wound healing

As for the application of ES, the most studied area is wound healing. Studies in animal models and clinical trials have generated relatively positive outcomes [182, 260, 261]. In the last few decades, the research field has expanded from studying endogenous EF to the artificial ES therapy for wound healing.

Studies have shown that applying an EF at the direction opposite to the wound-induced EF caused a nonhealing wound, while the opposite of this significantly increased wound closure [262]. ES can attract the cells necessary for the healing process, including neutrophils, macrophages, fibroblasts, and epidermal cells [33]. Studies have also shown that ES not only accelerated healing but also regenerated the tissue with increased tensile properties compared to the control [263]. Furthermore, ES can be beneficial in improving local perfusion, increasing temperature of the affected skin, and leading to a better wound healing [264]. ES also has been shown to promote angiogenesis, another important mediator of wound healing [265]. Of note, emerging evidences have suggested that a low frequency ES (< 10 Hz) has a higher efficacy in healing capacity than that of the ES at higher frequencies (> 50 Hz) [31]. DC [261], PC [72], and PEMF [266] have all been utilized in wound healing [29]. Although initial findings are encouraging, further study is warranted to fine tune the ES parameters for specific wound size and thickness to harness the benefits. Specifically, future study should investigate different EF intensities, frequencies, and the delays from initial injury, to adequately assess the most beneficial way that ES could be used in clinical setting.

Several ES-based wound dressings have been developed and used in clinic. POSiFECT® is one of the early products developed by Biofisica, Inc. [267]. It consists of a ring-shaped anode placed outside the wound and a small cathode placed at the center of the wound bed to direct the EF or current in the wound bed. The power is provided by an integrated battery module and a constant ES current is ensured through a control circuit. Procellera® is a wound dressing developed by Vomaris Innovations, Inc. [268]. This product utilizes a novel microcurrent-generating technology which produces electrical fields through a matrix of embedded microcell batteries. It has been shown that the Procellera® wound dressing has antibacterial effect against clinically relevant wound pathogens and could potentially reduce the risk of infection at the wound site.

I.5 Interactions of ES with keratinocytes

Endogenous EF is an important mechanism in guiding the migration of keratinocytes to the wound site and plays a pivotal role in promoting re-epithelialization.[182] Exogenous EF has been applied in keratinocyte culture to study its effect on the cells. According to the findings, it is likely that DC EF between two electrodes can direct the migration of keratinocytes toward cathode [269]. To summarize, 1) Migration of human keratinocytes in EF requires growth factors and is Ca^{2+}-dependent [270]; 2) EGFR relocalization and kinase activity are necessary for the directional migration of keratinocytes in DCEF [68]; 3) Response of keratinocytes to DCEF is dependent on differentiation status [271]; 4) Cyclic AMP-dependent protein kinase A plays a role in the directed migration of keratinocytes in a DCEF [272].

Studies have revealed the following mechanisms:

1) Following an ES, keratinocytes polarize and migrate, together with the activation of PI3 kinase which is polarized to the leading edge [272]. However, polarized phosphatase and tensin homolog (PTEN) were found at the opposite side of the migrating cells. This suggests that PI3 kinase and PTEN act as "compass molecules" in directional sensing [273]. During such a process, multiple cAMP signaling pathways were reported to regulate keratinocyte electrotaxis [274, 275].

2) EGFRs are redistributed asymmetrically after the application of DCEF on keratinocytes. EGFR/PI3 kinase/F-actin signaling can mediate keratinocyte electrotaxis [276, 277]. In fact, an extracellular electrical signal can be sensed by the EGFR on cell membrane, which leads to the activation of the PI3 kinase/Akt and mitogen activated protein (MAP) kinase signaling pathways. The transduction of electric signal then results in an asymmetric polymerization of intracellular F-actin and a directional migration of keratinocytes, which further promotes wound healing [68].

3) Genetic and pharmacological evidence revealed a two-molecule model involving the K^+ channel/Kir4.2 and the intracellular polyamines. Initially, a weak extracellular EF redistributes the positively charged polyamines, which then bind to K^+ channel Kir4.2 and consequently regulate the flux of K^+. The K^+ alteration then induces changes in membrane potential, osmolality, and the ionic environment, which consequently interacts with the previously established intracellular PI3K/AKT pathways and ultimately affects actin polymerization, membrane protrusion, and cell migration [176, 278, 279].

4) ES-inducible SIVA1 modulates p53 activities in proliferating keratinocytes, and exogenous ES affects p53/HDM2/SIVA1 axis leading to an increased proliferation during re-epithelialization [279].

Despite the evidences suggesting the implication of EF in keratinocyte activities, the precise physiological role of EF on keratinocytes and re-epithelialization mechanisms remains to be determined [280].

I.6 Conductive polymers (CPs)

A conductive scaffold provides a unique and attractive option for delivering ES to cells or tissues [281]. A conductive scaffold can be prepared by blending biomaterials such as polylactide (PLA), polycaprolactone (PCL) or polyurethane (PU) with conductive particles such as gold, silver, or graphene particles [282-286]. However, problems like inhomogeneous distribution of the conductive particles in the composite lead to non-reliable conductive properties that limit the widespread and successful use of such composites [287]. Conductive polymers, owning to their intrinsically electrical conductivity and biocompatibility, make ES more efficient and a great potential in tissue engineering and regenerative medicine [288].

I.6.1 General introduction of CPs

Conductive polymers are a surprising discovery. Traditionally, polymers or plastics are known as insulators which can be used to protect the electric wires from short circuits. However, this view was changed, as in 1970s Alan J. Heeger, Alan G. MacDiarmid and Hideki Shirakawa synthesized the halogen derivatives of polyacetylene (CH)x (PA) [289]. A conductivity of 3000 Siemens per meter (S m^{-1}) was measured for the iodine-modified PA, which was 10^9 times more conductive than the original PA and was higher than any previously known polymers. Following that, the fluorine-modified PA was prepared and was 10^{11} times more conductive than original PA [290]. As a comparison, Teflon has a conductivity of 10^{-16} S m^{-1} and copper 10^8 S m^{-1}. This discovery stimulated the synthesis of various conjugated polymers [291]. As the results, an entire class of polymers was found to possess electrical conductivity after the halogen treatment. The typical representatives include polyaniline (PANI), poly(p-phenylene vinylene) (PPV), polythiophene (PT) and its derivatives, PEDOT, and PPy. The common feature of the chemical structures of CPs is the polyconjugation in the π -system of their backbone, as shown in Figure I.13 [292]. The conductive forms of these polymers are obtained by means of doping and the doping is reversible

as a result of dedoping (removal of dopant) [293]. CPs can have the electrical properties similar to metals while retain the mechanical properties and processibility of conventional polymers, which makes them very attractive. Researchers and engineers from a variety of fields in science and technology have engaged in CPs studies and have found that CPs may have promising applications in energy storage, sensors, photoluminescent and electroluminescent materials, "artificial muscles", gas separating membranes, anticorrosive coatings, electromagnetic shielding, drug delivery, etc [292]. Because Heeger, MacDiarmid and Shirakawa opened up the field of CPs, they were awarded the Nobel Prize in Chemistry in 2000 [291].

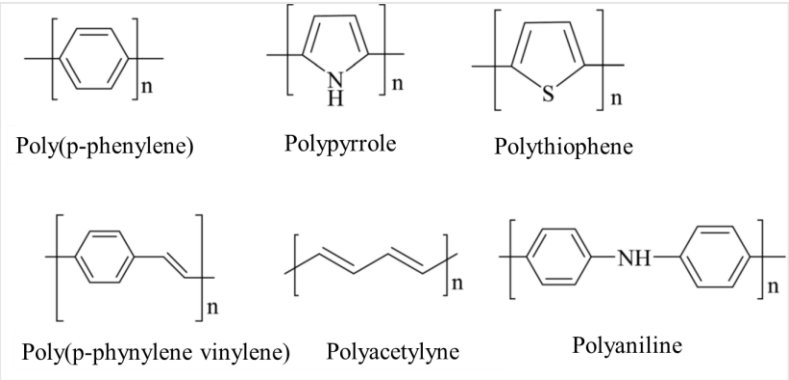

Figure I.13 Chemical structures of some common conductive polymers [292].

I.6.2 Conductive mechanism of CPs

Conductivity depends on the density of charge carriers and how fast they can move in the material. Based on classical band theory, the lowest unoccupied molecular orbital (LUMO) is called the *conduction band* and the highest occupied molecular orbital (HOMO) is called the *valence band.* The minimum energy spacing between LUMO and HOMO is called the *band gap (*ΔE), corresponding to the energy needed to create a charge pair with one electron in the upper (empty) band and one positive charge or "hole" in lower (filled) band [294], as shown in Figure I.14.

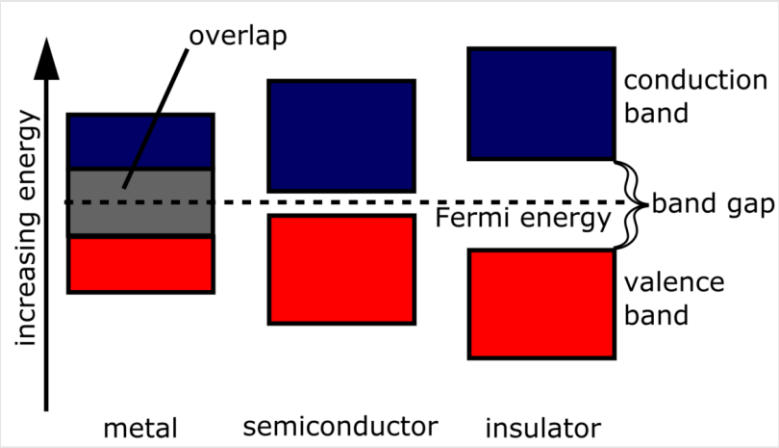

Figure I.14 Simple band gap diagram showing the difference between conductors, semiconductors, and insulators [295].

In polymers, when double bonds are added to molecules, every double bond contains a localized "sigma" (σ) bond which forms a strong chemical link, and additionally, a less localized "pi" (π) bond which is weaker. Alternating single and double bonds between the carbon atoms in polymer chains form conjugations. The conjugated double bonds behave quite differently from the isolated double bonds by that they act collectively. Hückel's theory [296] predicts that conjugated π electrons are spaced out rather evenly and delocalized over the entire chain. In other words, in conjugated π electron systems, all bonds are equal and ΔE becomes small because of this special system. However, for the overwhelming majority of conjugated polymers, their molecule structures are highly disordered, leading to the π -electrons that are insufficiently delocalized to facilitate charge transport [297]. Therefore, to delocalize π -electrons, a push factor is needed. Experiments have proved that delocalization can be achieved through treating the conjugated polymers with halogen, which is called "doping". There are p-doping (or oxidation) and n-doping (or reduction) [298]. The dopants are the chemicals injected into the material to form extra electrons or "holes" (a hole is a position where an electron is missing). A doped polymer is therefore like a salt. The roles of the dopants are to compensate the charge of the free charge carriers and simultaneously to enhance the probability of the charge transfer. Dopants can be single atom ions (Cl^-) or ionized small molecules (DMSO) or even polymers (PSS). By applying an EF, the dopants, which are also called counterions, can be made to diffuse from or into the material, causing the doping reaction to proceed backwards or forwards. In this way the conductivity can be switched off or on [293, 297-299].

In summary, a key property of CPs is the presence of conjugated double bonds along the backbone of the polymer. In addition, doping has to be introduced to form the mobile polarons and bipolarons along the polymer chain. Regulation of doping level provides a possibility to prepare polymers with a widely varied electrical conductivity, from the insulating form of the polymers in undoped state to the highly conductive ones in heavily doped state [298].

I.6.3 Polypyrrole (PPy)

PPy is by far the most extensively studied among the numerous CPs because of its easy synthesis, environmental stability, high electrical conductivity, and most importantly its excellent biocompatibility that endows PPy to be intensively investigated for biomedical applications [300].

PPy preparation. PPy is polymerized through the oxidation of pyrrole monomers (C_4H_5N), which can be carried out through electrochemical synthesis or chemical synthesis methods [301].

For **electrochemical polymerization**, the pyrrole monomer is oxidized on the anode in aqueous or non-aqueous medium, producing a thin PPy film on the electrode surface. The film adheres strongly to the electrode and is difficult to peel off, which limits its application [301].

Usually, a **chemical polymerization** is carried out by mixing pyrrole monomers and oxidant in a solution. The solvent can be water, methanol, benzene, chloroform, acetone, etc. The widely used oxidants are $FeCl_3$ and other salts of iron (III) and copper (II). The formed PPy is an opaque, brittle, and amorphous material, often in form of a black powder. The type of oxidant plays an important role in the yield and conductivity of the PPy. In some cases, the oxidant can be the dopant as well. The conductivity of PPy can range from zero to hundred S/cm [302]. Other factors, such as the choice of solvent, pyrrole concentration, duration and temperature of the reaction also influence the properties of the formed PPy [303, 304]. When pyrrole is oxidized by $FeCl_3$, the formed PPy will be automatically doped by Cl^- anions. The stoichiometric equation of the reaction is:

$$n\ C_4H_5N + (2+y)n\ FeCl_3 \rightarrow \left[(C_4H_3N)_n^{y+} nyCl^-\right] + (2+y)n\ FeCl_2 + 2n\ HCl$$

where y is the degree of PPy oxidation (doping level) and is determined from the Cl/N ratio. A Cl/N ratio equal to 0.25 has been identified [301].

As pristine PPy is brittle, insoluble and infusible [301, 303, 305], in order to have sufficient strength and ductility for practical use, polymerizing pyrrole inside the matrix of other polymers, *i.e.*, PVA,

PLA, PU, or nanoporous cellulose gels, etc. [306-313], has been used to form the conductive composites with enhanced mechanical properties. Another technique is to polymerize pyrrole on surface of the fabrics, *i.e.*, fabrics made of PET, PLA, PLGA, PCL and PVDF, etc. [24, 91, 312, 314-322], to produce a PPy coated fabric. The formed materials combine the conductive properties of PPy and the mechanical properties of the host polymer. However, for such heterogeneous polymer systems, the instability caused by the dissipative structures is a problem, because the heterogeneous material promotes the spread out of inelastic strains over the whole macroscopic sample [323, 324].

Our research group pioneered and conducted a great deal of research about PPy scaffold and PPy substrate-based ES for regenerative medicine [23, 72, 85, 86, 261, 325-328]. Recently, a soft pristine PPy membrane was successfully prepared, showing unique advantages as a substrate to support cell growth and to mediate ES [329]. However, such a membrane is difficult to manipulate because of its weak mechanical strength.

I.7 Aims of the study

I.7.1 Research question

Based on the above-mentioned background, it's easy to raise my research question, which is "can we reinforce the soft PPy membrane without losing its softness and conductivity, and use it as a material platform to deliver electrical stimulation to soft tissues like skin and to skin cells?" For this reason, this study is to reinforce the soft PPy membrane, to use the reinforced PPy membrane to deliver ES, and to investigate the keratinocyte behaviors under ES treatment. The overall study will pave the way for the soft PPy membrane to be employed as a tool in ES-assisted therapy for wound healing.

I.7.2 Hypothesis

1. The soft PPy membrane can be reinforced with electrospun polymer fibres without losing its electrical property and cytocompatibility.

2. ES mediated by the soft PPy membrane can trigger ERK1/2 signaling pathway to modulate the release of cytokines and growth factors by the keratinocytes, in favor of wound healing.

I.7.3 Objectives

The principal objective is to prepare a soft PPy membrane as a suitable substrate to deliver ES to keratinocyte, and to study how ES activates keratinocytes at both cellular and molecular levels in the context of improving wound healing. The specific objectives are:

1. To develop a new conductive substrate by reinforcing a soft PPy membrane with electrospun polymer fibers without affecting the electrical property, surface chemistry and cytocompatibility of the membrane.

2. To investigate in vitro the effects of ES on human skin keratinocytes in terms of the cell proliferation, secretion of growth factors and cytokines, and the signaling pathway.

I.7.4 Research design

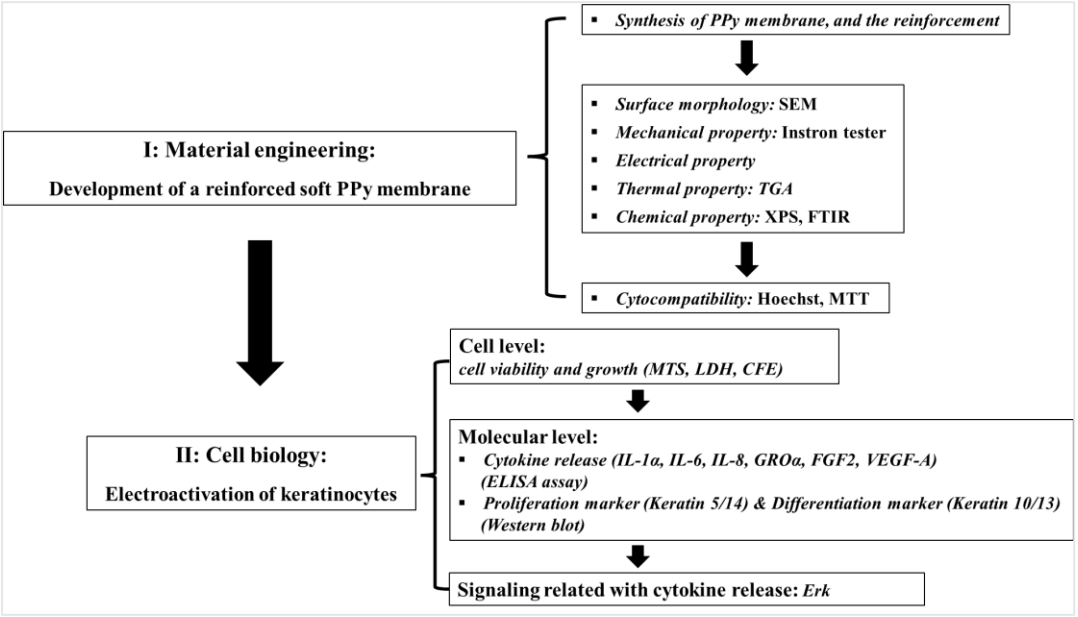

Figure I.15 The schematic of research design.

Considering the brittleness and the poor handling property of the soft PPy membrane, the mechanical property of this membrane must be improved before it can be used for cell culture. Considering that the soft PPy membrane is to be used as an ES scaffold, only biocompatible polymers should be selected as the reinforcing materials, and cytotoxicity must be excluded during the process. This chapter therefore studies use PU and PLLA fibers to reinforce the soft PPy membrane by an electrospinning technique. The reinforced PPy membrane keeps the conductivity of PPy, is not cytotoxic, and therefore can be used as an ES scaffold.

Chapter 1: To engineer a reinforced biocompatible polypyrrole membrane for biomedical applications

A biocompatible polypyrrole membrane for biomedical applications

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Résumé

Le polypyrrole (PPy) est le biomatériau conducteur le plus étudié. Cependant, en raison de sa rigidité intrinsèque, le PPy n'a été utilisé que sous la forme d'un composite ou d'un revêtement mince. Ce travail présente une membrane souple à base de PPy pur, renforcée de manière synergique par des fibres de polyuréthane (PU) et d'acide poly-L-lactique (PLLA) électrofilées. Ce renforcement particulier rend non seulement la membrane PPy facile à manipuler, mais il empêche également la membrane de se déformer dans un environnement aqueux. Des tests de pelage et des tests mécaniques ont confirmé la forte adhésion des fibres et l'augmentation de la résistance à la traction de la membrane renforcée. La conductivité électrique de surface et la stabilité électrique à long terme ont été testées, montrant que ces propriétés n'étaient pas affectées par le renforcement. La morphologie et la chimie de la surface ont été analysées par spectroscopie électronique à balayage (SEM), spectroscopie photoélectronique à rayons X (XPS) et spectroscopie infrarouge à transformée de Fourier (FTIR). La stabilité thermique du matériau a été étudiée par analyse thermogravimétrique (TGA). Enfin, l'adhésion et la prolifération de kératinocytes de peau humaine sur la membrane ont été évaluées par coloration de Hoechst et par le test au bromure de méthylthiazolyldiphényl-tétrazolium (MTT). En conclusion, cette membrane s'avère être le premier biomatériau conducteur souple à base de PPy pouvant être utilisé de manière pratique. Sa conductivité électrique et sa cytocompatibilité promettent une utilisation biomédicale élargie de cette membrane à base de PPy.

Abstract

Polypyrrole (PPy) is the most widely investigated electrically conductive biomaterial. However, because of its intrinsic rigidity, PPy has only been used either in form of a composite or a thin coating. This work presents a pure and soft PPy membrane that is synergically reinforced with the electrospun polyurethane (PU) and poly-L-lactic acid (PLLA) fibers. This particular reinforcement not only renders the originally rather fragile PPy membrane easy to manipulate, it also prevents the membrane from deformation in an aqueous environment. Peel and mechanical tests confirmed the strong adhesion of the fibers and the significantly increased tensile strength of the reinforced membrane. Surface electrical conductivity and long-term electrical stability were tested, showing that these properties were not affected by the reinforcement. Surface morphology and chemistry were analyzed with scanning electron spectroscopy (SEM), X-ray photoelectron spectroscopy (XPS), and Fourier transform infrared spectroscopy (FTIR). Material thermal stability was investigated with thermogravimetric analysis (TGA). Finally, the adhesion and proliferation of human skin keratinocytes on the membrane were assessed by Hoechst staining and the methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay. In conclusion, this membrane proves to be the first PPy-based soft conductive biomaterial that can be practically used. Its electrical conductivity and cytocompatibility promise a wide range of biomedical applications.

1.1. Introduction

Polypyrrole (PPy) is an inherently conductive polymer formed through consecutive couplings of the oxidized heterocyclic pyrrole (Py) monomers and oligomers [1]. Unlike polyacetylene, PPy is hardly crystalline; and instead of forming a planar structure, PPy grows and aggregates layer by layer [2, 3], making it low in tensile strength, with a lack of plasticity and elasticity, and poor in processability [4].

Meanwhile, due to its electrical conductivity, environmental stability and excellent biocompatibility [5], PPy has been intensively investigated as a biomaterial [6]. Considering its potential applications in electrical stimulation including in clinic therapies [7-11], PPy also becomes an interesting scaffolding material or an interface to mediate electrical stimulation to cells or tissues, such as in cancer therapy or tissue regeneration [12, 13]. All of these, however, are based on the promise to have a processable PPy scaffold with adequate mechanical strength [14].

Abundant research work has concentrated on improving the mechanical properties of PPy by combining it with supporting polymers [6, 15]. A commonly used method is to blend PPy particles with supporting polymers including *in situ* polymerization of PPy in a polymer solution, followed by solution casting and solvent evaporation to obtain a composite PPy membrane. Polymers that are frequently used include polylactide (PLA) [15], polyurethane (PU) [16-18], chitosan [19] and polycaprolactone (PCL) [20]. With this approach, normally only a small percentage of the PPy particles are dispersed in the supporting polymer, which should be adequate to make the composite conductive but not too high to make the mechanical property weak. The key point of this technique is to homogeneously distribute the PPy particles and, at the same time, form a PPy network to conduct electricity. Another widely used method is surface modification [21-23]. For example, soaking a polyester fabric in pyrrole monomer solution followed by oxidation polymerization can lead to a conductive textile, with a thin layer of PPy coated on the individual fibers if pyrrole is not excessive to fill the microporous structure.

Other nonconventional technologies have also been used. For example, electrospinning a polymer/PPy solution can generate fibrous conductive scaffolds [24, 25]. *In situ* vapor-phase polymerization of pyrrole monomers in cellulose gels followed by supercritical drying can produce a conductive aerogel [26]. A flexible PPy/cellulose composite membrane was prepared through

electrochemical polymerization by firstly sputter-coating a paper with platinum [27]. The work of PPy and its composites in biomedical applications have been extensively reviewed recently [28, 29].

However, the above-mentioned methods have two major limitations. In the approach of using PPy particles as fillers, conductivity of the composite is significantly lower than that of the pure PPy, for the supporting polymers are normally insulators. In the second approach, *i.e.*, surface coating, although the thin PPy coating can be as conductive as the pure PPy, this conductivity deteriorates rapidly once used in an aqueous environment due to dedoping [3]. The lowering of conductivity in either approach can be as significant as 3 to 4 orders of magnitude.

The free standing soft PPy membrane synthesized *via* a template-assisted interfacial polymerization technique largely addressed these two problems [30]. This PPy membrane has a distinctive physical structure that, for the first time, makes the pure PPy membrane soft and flexible. However, the tensile strength of this membrane is poor, with an elastic modulus of 3.4 MPa and an ultimate elongation 2.5%, making it easily damaged if not handled properly. Because free standing pure PPy membranes are hardly available and non-extendable, the tensile strength of pure PPy in literature is very rare. One group reported a PPy foam with a tensile strength of 18 kPa at 3.3% elongation [31].

In this study, we aimed to improve the mechanical strength of the free-standing soft PPy membrane without losing its other properties. For that purpose, polyurethane (PU) and poly(D,L-lactide) (PLLA) were sequentially electrospun onto the PPy membrane. The final membrane displayed unchanged electrical conductivity, significantly improved tensile strength and handling property, and an excellent cytocompatibility.

1.2. Materials and methods

1.2.1 Materials

Pyrrole (98%, Alfa Aesar), ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$, 98%, Sigma-Aldrich), methyl orange (MO, ACS reagent, dye content 85%, Sigma-Aldrich), chloroform ($CHCl_3$, HPLC grade, Fisher Scientific), PU (Tecoflex™ SG-80A, Thermedics), PLLA (intrinsic viscosity 1.3, Hycail). All chemicals except pyrrole were used as received. Pyrrole was distilled and kept at 4 °C before use.

1.2.2 Synthesis of PPy membrane

PPy membranes were synthesized according to the technique published in our previous work [31]. Briefly, 3 mL pyrrole was dissolved in 150 mL $CHCl_3$ and maintained at 4 ℃ for at least 1 h before use. The amount of 18.2 g of $FeCl_3 \cdot 6H_2O$ and 5 mM of MO were simultaneously dissolved into 320 mL of deionized water, and stirred for 30 min to obtain a $FeCl_3$/MO complex. The interfacial polymerization started when the $FeCl_3$/MO complex was transferred onto the top of the cold pyrrole chloroform solution in a 15 cm in diameter beaker. After 48 h polymerization at 4 °C, the PPy membrane was collected and washed sequentially with ethanol and deionized water to colorless. A HCl solution (1.67 mol L^{-1}) was dropped into the washing solution and the absence of pink color indicated the complete removal of $FeCl_2$ and MO. Then the membrane was dried at ambient temperature and named PPy for later use.

1.2.3 Strengthening PPy membrane with electrospun fibers

After trying 10, 12, 15 and 18 wt% concentrations, 15 wt% was found most appropriate to spin both PU and PLLA fibres using a handheld electrospinning device (Bona, Qingdao, China). The PU and PLLA solutions of 15 wt% were separately prepared by adding solid polymer pellets to $CHCl_3$, and then stirred at room temperature overnight. A volume of 1.5 ml of the polymer solution was then fed into a syringe that was then loaded to the handheld electrospinning device. Fibres were spun through a #25 gauge needle to cover 241 cm^2 of PPy surface (*i.e.*, 0.93 mg cm^{-2}) at a feeding rate of about 2.25 mL h^{-1} under a DC voltage about 9 kV at 25 °C and 65% humidity. The bubble side of the PPy membranes [30] was used as the collector, which was 25 cm away from the needle. To spin composite fibers, the PU fibers were firstly spun, followed by the PLLA fibers, as illustrated in Figure 1.1. The fibre-reinforced PPy membrane was then placed in an oven at 40 ℃ overnight to evaporate $CHCl_3$. The reinforced membranes herein were identified as PPy-PU, PPy-PLLA and PPy-PU/PLLA.

In addition, the dried original and reinforced PPy membranes were immersed in deionized water for 7 day with the water refreshed twice a day to further remove impurities and reduce cytotoxicity. The membranes were subsequently dried naturally in the fume hood. The membranes, after 7 day wash, were named as wPPy, wPPy-PU, wPPy-PLLA, and wPPy-PU/PLLA.

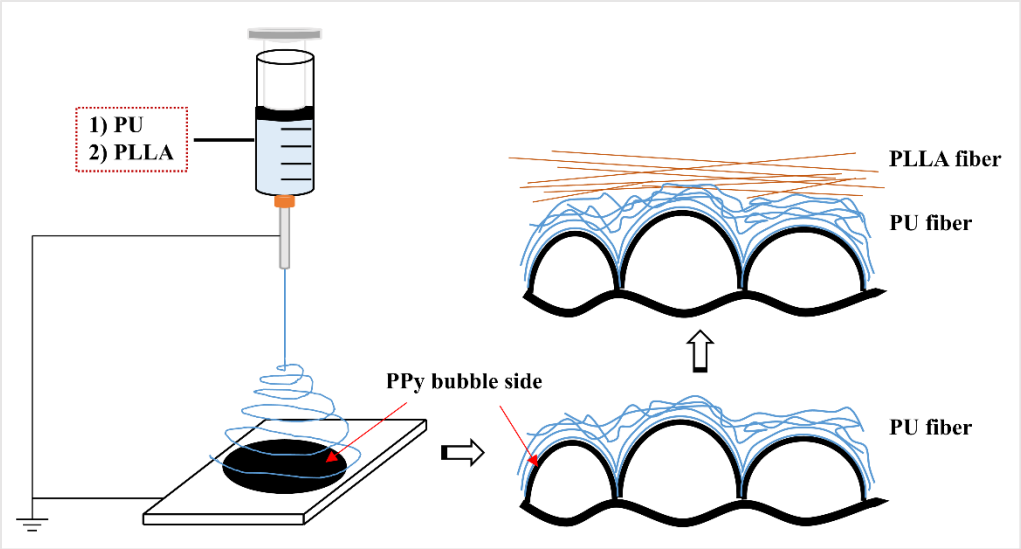

Figure 1.1 Schematic illustration of how the electrospun fibers are assembled on top of the bubble surface of PPy membrane.

1.2.4 Characterizations

1.2.4.1 Surface morphology

A scanning electron microscope (SEM, model JSM-6360LV, JEOL, Tokyo, Japan) at an accelerating voltage of 20 kV was used to observe the morphological microstructures of the membranes. Specimens were sputter-coated with gold in a sputter coater (Fison Instruments, Polaron SC500, Uckfield, UK). Photomicrographs of various magnifications were taken from the representative parts of the surface and cross section.

1.2.4.2 Peel test

To measure the adhesion strength of the PU and PLLA fibers to the PPy surface, peel tests were conducted on the bubble side of the membrane. A circular PPy membrane was cut from the middle into two halves, to ensure the two membrane specimens being synthesized from the same batch of experiment. These two semicircular membranes were electrospun with either PU or PLLA fibers. Before electrospinning, a 5 mm wide of the straight edge of the specimen was covered with a piece of paper, to shield the edge from landing of the fibers. During electrospinning, the fibers landed on the surface of the membrane and the paper, as illustrated in Figure 1.4A. After evaporation of the solvent in an oven at 40 °C overnight, the fibers were slowly peeled off from the membrane surface by pulling the paper at a 45° angle against the horizontal membrane surface. The fiber

adhesion strength was measured and photographed by the two outcomes, *i.e.*, either the fibers were peeled off without damaging the membrane, or the membrane was broken while peeling off the fibers. The peeling test was repeated 5 times for each type of fiber.

1.2.4.3 Tensile strength measurement

An Instron 5848 MicroTester (Instron, Norwood, MA, USA) was used for the tensile strength test. The specimens were cut into the shape of a "dog-bone", with 7 mm width at two ends and 3.5 mm x 16 mm in the middle. The distance between two gauges was 13 mm. The stretch was carried out at a rate of 1.0 mm min^{-1} till failure. For each sample, data were collected at least from 5 specimens broken in the middle. The thickness of the samples was measured using a thickness gauge under the pressure of 19.76 kPa (MTG-DX2, Rex Gauge Company, Buffalo Grove, IL, USA).

1.2.4.4 Electrical conductivity

The surface resistance (*Rs*) of the non-reinforced side of the membranes was measured and averaged from at least 10 measurements at randomly selected locations using the Jandel multi-height four-point probe (Jandel Engineering Ltd., Linslade, Beds, UK) at room atmosphere. The four probes have a separation of 1 mm and a diameter of 100 μm. The surface electrical conductivity (σ) was calculated referring to the publication from Smits [32].

1.2.4.5 Electrical stability

The electrical stability of the membranes under cell culture conditions was investigated using a home-made multi-well electrical cell culture plate [8]. The wPPy-PU/PLLA membrane was mounted into the plate, immersed in culture medium, and kept in a standard cell culture incubator. The two ends of the membrane outside the culture well were applied with a 200 mV mm^{-1} potential gradient, with the current recorded *versus* time for 240 h using a Keithley 2700 Digital Multimeter/Data Acquisition System (Keithley Instruments, Cleveland, OH, USA). Eight tests were performed for this experiment.

1.2.4.6 FTIR test

The Fourier transform infrared (FTIR) spectra of the membranes were recorded with a Nicolet Magna-IR 550 spectrophotometer (Nicolet Instrument, Madison, USA) at the attenuated total reflectance (ATR) mode. To do so, the specimens were pressed against a hemispherical silicon

crystal and scanned 64 times between 500 and 4000 cm^{-1} at a resolution of 4 cm^{-1}. Considering the small thickness of the membrane (*ca.* 0.7 μm) [30] and the sampling depth of the silicon crystal (*ca.* 0.8 μm at 45 degree and 1000 cm^{-1}), this analysis should be considered bulk even if it was done at ATR mode. The ATR-FTIR test was conducted on the fiber-free side of the membranes.

1.2.4.7 XPS analysis

The X-ray photoelectron spectrometer (XPS, Perkin-Elmer PHI model 5600, Eden Prairie, MN, USA) was used to examine the surface elemental composition and chemical valence states of the membranes. The survey scans were obtained using a monochromatic aluminum source at 1486.6 eV, and the high-resolution spectra were achieved *via* a standard magnesium source at 1253.6 eV. The XPS analysis was performed on the fiber-free side of the membranes.

1.2.4.8 Thermogravimetric analysis

The thermogravimetric analyzer TGA/SDTA 851e (Mettler-Toledo, Mississauga, Ontario, Canada) was used to measure the thermal degradation of the membranes by heating at a rate of 20 °C min^{-1} from 25 to 600 °C in the atmosphere of nitrogen flowing at a rate of 20 ml min^{-1}.

1.2.4.9 Cytocompatibility

The wPPy-PU/PLLA membranes were cut into circular specimens according to the size of the 24-well culture plate and sterilized with ethylene oxide gas following standard industrial procedures. Human skin keratinocytes (HaCat, Cedarlane CELLutions Biosystems, Burlington, ON, Canada) were seeded on the fiber-free side of the membranes at 1×10^5 cells per well for cell morphology analysis and at 2×10^5 cells per well for cell proliferation analysis. The cells were cultured for 24, 48 and 72 hours and then either stained with Hoechst dye 33342 (Riedel de Haen, Seele, Germany) for adhesion observations or processed for MTT assay (Sigma-Aldrich, Canada) for viability assessment. The cell culture experiment was triplicate at each time point. For MTT assay, four measurements were performed for each experiment.

For Hoechst staining, the cells were first washed three times with PBS and then fixed with a mixture of methanol in acetone (3: 1) for 10 min. After, the cells were incubated with a solution of 2 mg mL^{-1} of Hoechst 33342 in PBS for 15 min at room temperature and then washed with PBS. The cells were finally observed under an epifluorescence microscope (Axiophot, Zeiss,

Oberkochen, Germany), and photographed. The cells cultured on glass slides were used as the control group.

To do MTT assay, the prepared 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg mL^{-1}) was stored at 4 °C prior to use. The cells were refreshed with the new medium containing 10% (v/v) MTT and cultivated in an incubator for 4 h without light. The supernatant was then carefully removed and 2 mL of 0.04 N HCl in isopropanol (lysis solution) was added. Fifteen minutes later, 200 mL of the solution was transferred in triplicate from each well to a 96-well flat bottom plate. Absorbance of the formazan at 550 nm was determined using an ELISA reader (Model 680, BioRad Laboratories, Mississauga, ON, Canada). Cells cultured in standard 24-well plate were used as control.

1.2.5 Statistical analysis

All data were presented as mean ± standard deviation ($n \geq 3$) when appropriate. Student t-test and Two-Way Analysis of Variance (ANOVA) were performed to compare the variations between two experiments or among a group of experiments, respectively. $p < 0.05$ was considered statistically significant.

1.3. Results and discussion

1.3.1 The synergy between PU and PLLA fibers

The surface of the soft PPy membrane has two distinct topographies: a flat nanotube side and a porous bubble side (Figure 1.2). Since the flat surface after removal of the nanotubes was used for cell culture, the reinforcement could only be done on the bubble side. The size and shape of the bubbles vary, ranging from 1 mm to 0.1 mm. The bubbles can be closed or open (Figure 1.2B). Such a surface topography significantly reduced the contact area between the PPy bubbles and any non-compliant fibers. Consequently, in this work we used two types of polymer fibers, *i.e.*, PU and PLLA. The PU fibers are soft and compliant as compared with the PLLA fibres, so can better follow the surface topography of the PPy bubbles to achieve a large adhesion area. However, the PU fibers can absorb a small amount of water and became slightly swollen in culture medium, forcing the soft PPy membrane to deform. Therefore, the rigid PLLA fibers were added to strengthen and stabilize the PU reinforced PPy membrane. As shown in Figure 1.3, the PU fibers

landed on the bubble surface and deformed according to the surface topography (Figure 1.3F&H), forming an intimate contact to PPy. In comparison, the PLLA fibers were straight and spanned the neighboring bubbles without following surface contour (Figure 1.3C&G). When electrospinning PU and then PLLA on the membrane, the composite fibers showed a strong adhesion to the PPy membrane, where the compliant PU fibers acted as an adhesive primer between the PPy and PLLA (Figure 1.3I, Figure 1.2D-F). The thickness of either PU or PLLA layer was estimated to be between 50 to 70 □m.

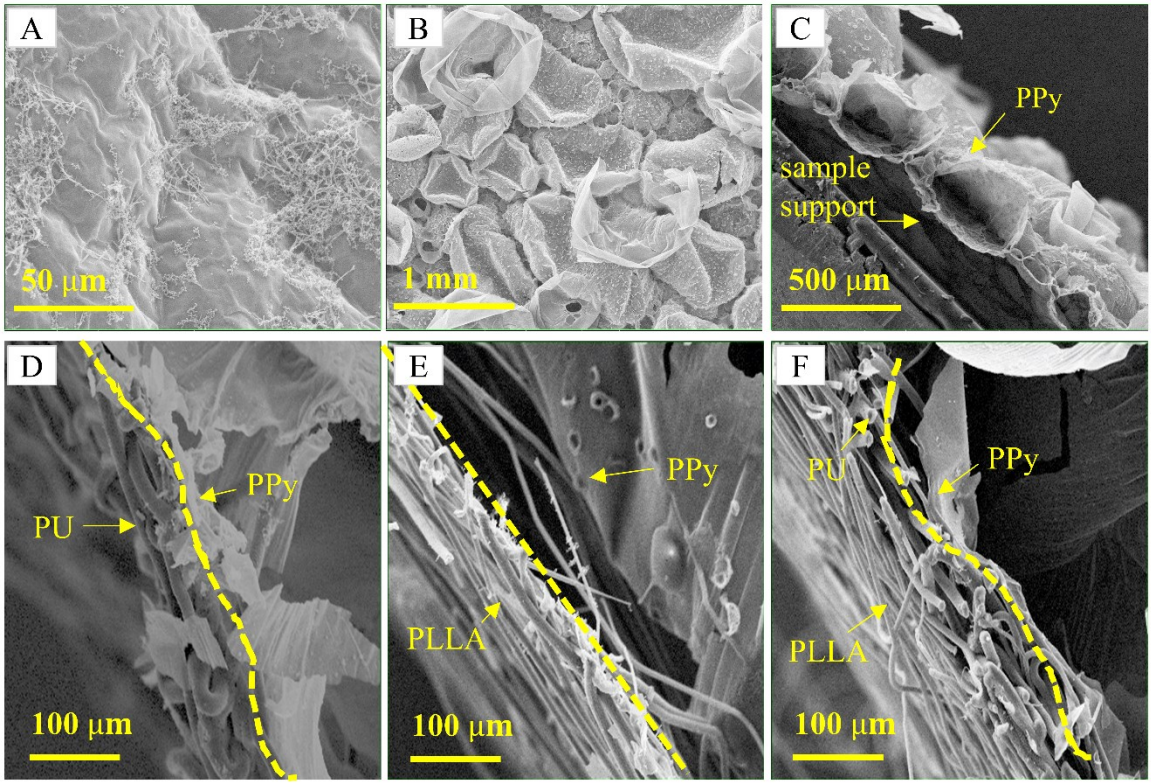

Figure 1.2 SEM of the original PPy membrane (A-C) and the reinforced PPy membrane (D-F). (A) nanotube side; (B) bubble side; (C) cross section; (D) the good attachment of the compliant PU fibers to the PPy; (E) the straight PLLA fibers detached from the membrane; and (F) the integration of the composite fibers to the PPy surface.

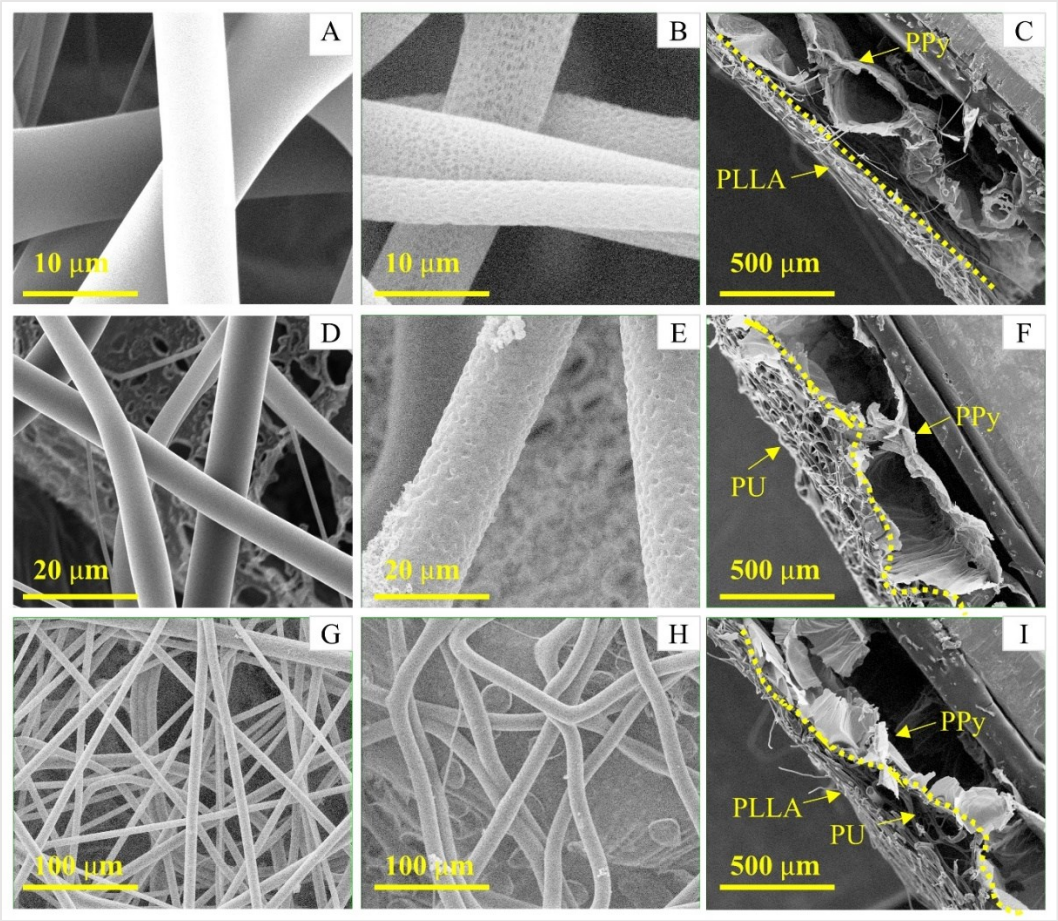

Figure 1.3 SEM images of the fibers and the cross section of the reinforced PPy membranes. (A–C) PLLA reinforced membrane, showing the PLLA fibres before (A) and after (B) wash, the cross-section of the washed membrane after wash. (D-F) PU reinforced membrane, showing PU fibres before (D) and after (E) wash, and the cross-section of the washed membrane. (G–I) PLLA/PU reinforced and washed membrane wPPy-PU/PLLA, showing the straight PLLA fibres (G), compliant PU fibres (H), and the cross-section of the membrane (I).

Safety must be adequately ensured for biomaterials. Reducing cytotoxicity through washing is a critical step for the as-prepared membranes. After wash, tiny dents appeared over the surface of the PLLA and PU fibers, as shown in Figure 1.3B&E. The probable causes include the extraction of residual chloroform that was not completely evaporated through conventional drying, and the hydrolytic degradation of the PLLA fiber during washing [33].

Peel test showed that the PLLA fibers were very easily peeled off the PPy membrane, whereas the PU fibers adhered strongly to the PPy membrane making it impossible to peel off the PU fibers without breaking the PPy membrane (Figure 1.4B&C). These results are consistent with the SEM observations, *i.e.*, the PU fibers were better integrated with the PPy bubbles. The Tecoflex™ SG-

80A is a polyether-based aliphatic thermoplastic polyurethane, of which the electrospun fibers were highly elastic and compliant. The PU fibers were also very sticky to the PPy probably because of the urethane groups that might have formed hydrogen bonds with the PPy. The PLLA, on the other hand, is a polymer with high crystallinity, which imposes rigidity and is compatible with the PU fibers. Consequently, the PU fibers stuck to both PPy and PLLA firmly, like a glue.

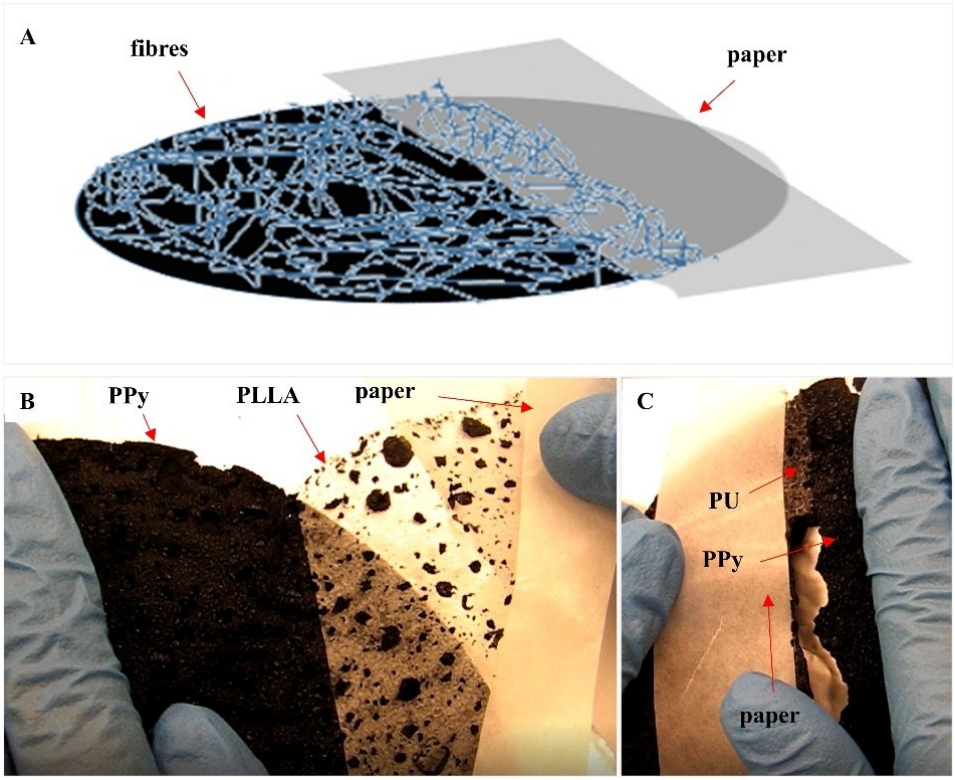

Figure 1.4 Peel test of the electrospun fibers on PPy membrane, showing the weak adhesion of the PLLA fibers and the strong adhesion of the PU fibers. (A) illustration of the peel test; (B) PLLA fibers were easily peeled off without damaging the PPy membrane; (C) PU fibers cannot be peeled off without breaking the PPy membrane.

To test the usability, the PU/PLLA reinforced PPy membranes and the non-reinforced (naked) PPy membranes were pressed with screws to the bottom of a home-made cell culture device (Figure 1.5A) and then released, mimicking the manipulations in a real cell culture experiment. The naked PPy membranes were found broken while the reinforced membranes were intact (Figure 1.5B&C). Because of the flexibility of the PU fibers and rigidity of the PLLA fibers, the reinforced PPy membrane exhibited a sufficient resistance to deformation and pressure.

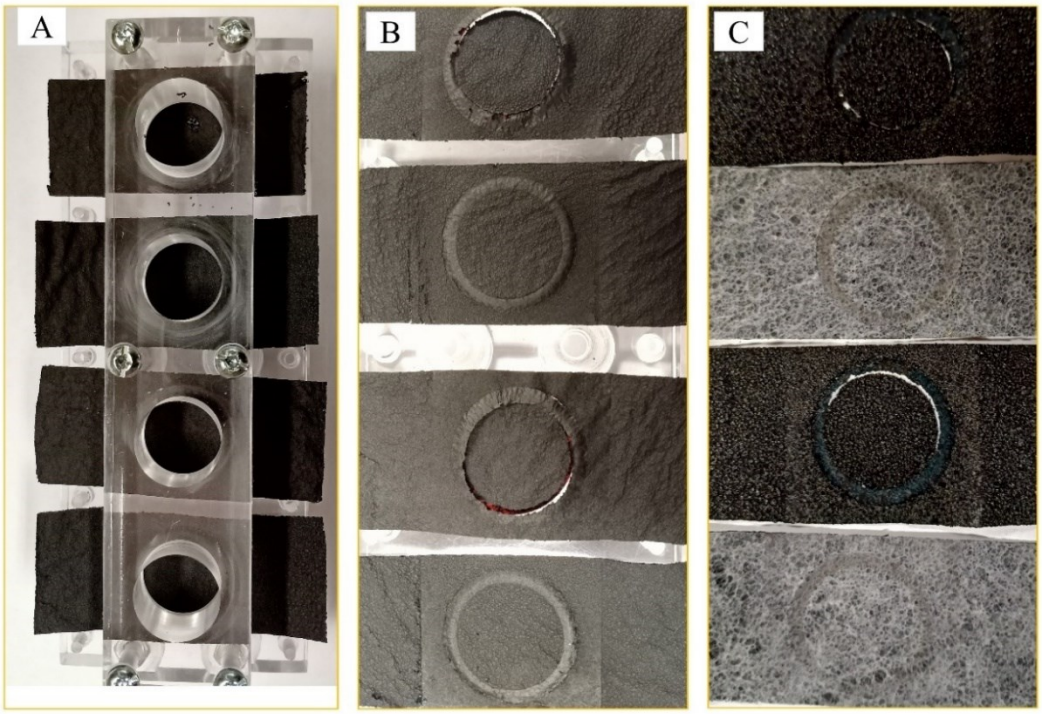

Figure 1.5 Usability test of the PU/PLLA strengthened PPy membranes in comparison with the non-reinforced membranes, showing the intact reinforced membranes (2nd and 4th rows) and the broken non-reinforced membranes (1st and 3rd rows). (A) Membranes assembled in the home-made cell culture device; (B) Membranes disassembled from the cell culture device; (C) Reverse side of the membranes. The white appearance of the 2 membranes in column C is because of the electrospun fibers.

1.3.2 Mechanical and handling properties

In general, the stress-strain curves of the PPy membranes are linear and show very low stress and strain at failure, which is expectable for such a brittle material (Figure 1.6). Interestingly, the washing procedure significantly reduced the ultimate strain (4.13 *vs.* 2.78%, $p < 0.01$) and stress (693 *vs*. 377 KPa, $p < 0.05$) of the PPy (Table 1.1). Knowing that PPy is not vulnerable to hydrolysis, we believe that such a drop in mechanical property is likely because of the leaching of dopants and possibly other small molecules such as oligomers out of the PPy, causing the PPy less compact and generating weak points. These small molecules also could have acted as plasticizers that render the PPy slightly more ductile. The differences in the strain and stress at break of the PPy are insignificant between the PPy and PPy-PU groups before wash ($p > 0.5$), indicating that the electrospinning of PU did not change the ductility of the PPy membrane. However, after wash, the PPy-PU membranes showed the largest decline in stress at break, *i.e.*, 544.09 vs. 229.25 KPa ($p < 0.01$). This is because that the PU fibers absorbed water and expanded, which stretched and

damaged the PPy membrane. In fact, the PPy-PU membranes were found deformed during washing. Importantly, such a PU fiber-caused membrane deformation was not observed among the PPy-PU/PLLA membranes during washing. Apparently, the rigid PLLA fibers prevented such a deformation as well as the damage to the PPy.

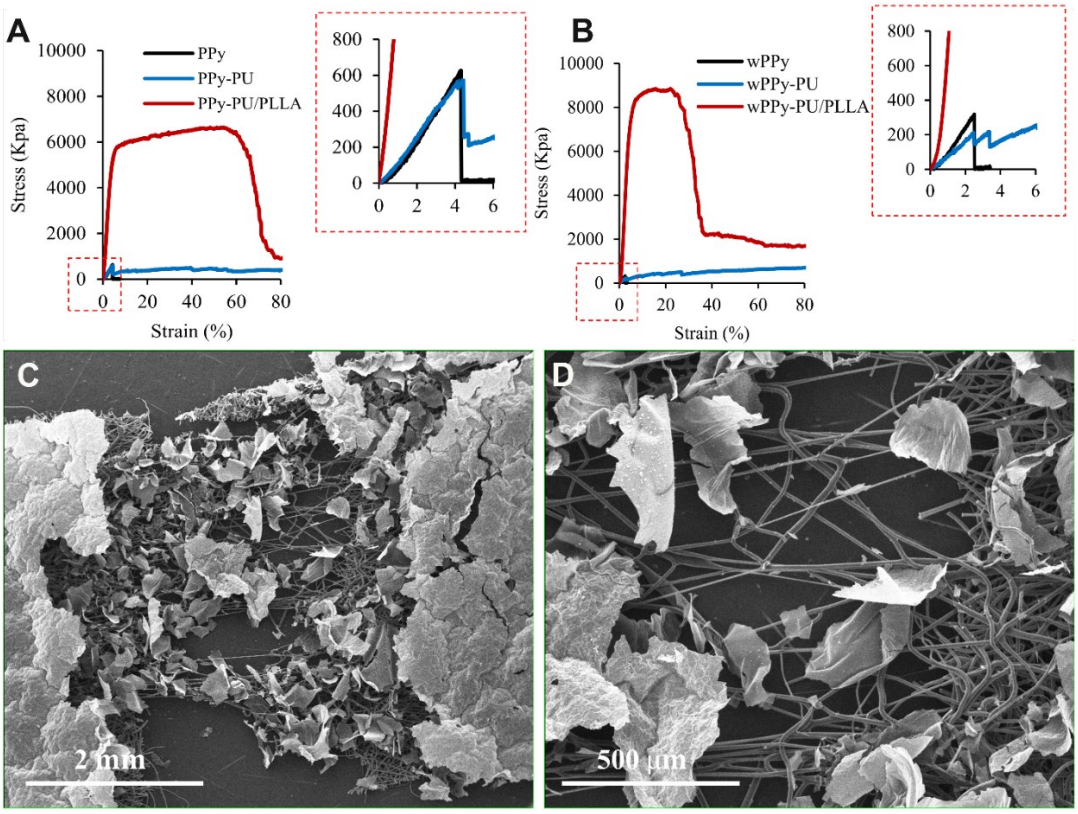

Figure 1.6 Stress-strain curves of the membranes, before (A) and after (B) 7 day wash, and the SEM photos of the stretched and broken wPPy-PU/PLLA specimen at low (C) and high (D) magnifications.

For the reinforced membranes, the high stress and strain reflect the property of the electrospun fibers rather than that of the PPy. Even the reinforced membranes recorded a much higher stress and strain, the PPy membrane still broke at a low strain. As shown in Figure 1.6, the failure of PPy can be clearly identified for the PPy and PPy-PU membranes. For the PPy-PU/PLLA membrane, the break point of the PPy was shield by the strong PLLA fibers and became undetectable. Table 1.1 only lists the stress and strain of the PPy at failure because these are the data that really mater. In this work, the role of the fibers is to prevent the strain of the PPy membrane from reaching the strain of failure during manipulation, not to make a strong and elastic composite membrane. The PLLA fibers showed a yield point followed by a large plastic deformation before failure, which is

normal. They became stiffer after wash likely because of the better organized crystalline structures and the complete removal of the residual chloroform, which is supported by the SEM results. The PU fibers on the other hand were very elastic and recorded a large deformation at low stress.

Table 1.1 Mechanical properties of PPy in different membranes

Condition	Membrane	Strain at break (%)	Stress at break (KPa)
Before wash	PPy	4.13±0.41	692.82±79.60
	PPy-PU	4.30±0.35	544.09±21.96
	PPy-PU/PLLA	-[a]	-[a]
After wash	wPPy	2.78±0.32	376.90±98.02
	wPPy-PU	2.77±0.18	229.25±32.91
	wPPy-PU/PLLA	-[a]	-[a]

[a] undetectable

1.3.3 Electrical conductivity

Table 1.2 Surface electrical conductivity

Condition	Membrane	Surface electrical conductivity (S cm^{-1})
Before 7 day wash	PPy	0.161±0.016
	PPy-PU/PLLA	0.109±0.012
After 7 day wash	wPPy	2.41E-04±2.58E-05
	wPPy-PU/PLLA	2.17E-04±1.73E-05

The surface conductivity of the membranes is presented in Table 1.2. The influence of wash and electrospinning on surface conductivity was analyzed through two-way ANOVAs. The 7 day wash significantly reduced the surface conductivity of both PPy ($p < 0.01$) and PPy-PU/PLLA ($p < 0.01$) membranes. In fact, the surface conductivity dropped by three orders of magnitude after wash. The reason is the leaching out of the dopants from PPy, resulting in the decay of conductivity. The difference between the PPy and PPy-PU/PLLA groups before wash was significant ($p < 0.01$), meaning that chloroform in the electrospinning process can affect PPy conductivity. However, this small difference is negligible in terms of conductivity, particularly for conductive polymers. This difference disappeared after wash ($p > 0.05$), which marks the effectiveness of washing in comparison of conventional drying in removing trace chloroform from polymers.

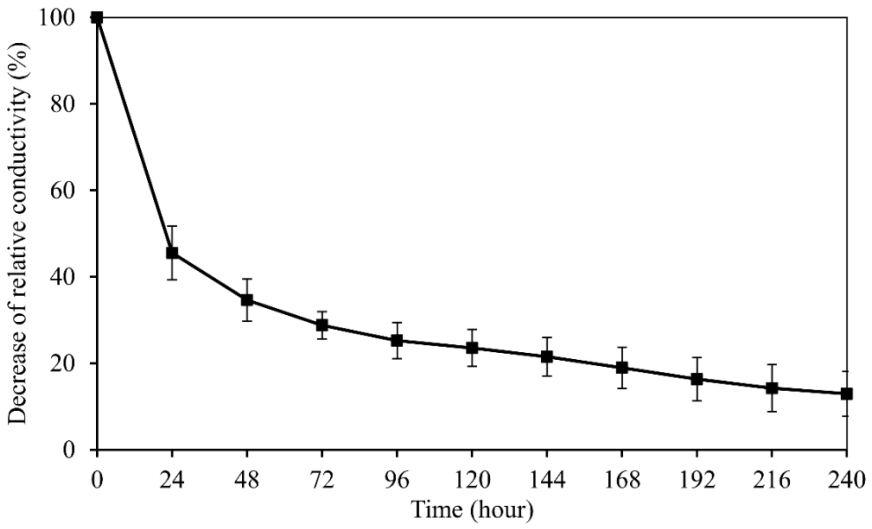

Figure 1.7 Electrical stability of the wPPy-PU/PLLA membrane

The peak voltage gradient at the wound edge was reported 140 mV mm^{-1} in skin and 42 mV mm^{-1} in cornea [34, 35]. So, the electrical stability of the wPPy-PU/PLLA membranes was tested in a 200 mV mm^{-1} electrical field. As shown in Figure 1.7, after a sharp decline in the first 24 h, which was about 65% of the initial conductivity, the conductivity continued to decrease but in a slow and linear fashion, with 13% of the initial conductivity retained after 10 days. The resistivity at this point was at the level of 10^3 ohm cm, which is still similar to that of the living animal tissues[36]. The first exponential decrease was due to the de-doping of anions from the PPy to the aqueous environment, a process enhanced by the reductive electrical potential. However, at same time the anions in the medium could diffuse back to re-dope the PPy. The second relatively stable stage in Figure 1.7 is attributed to the dynamic de-doping and re-doping processes. Knowing the electrical behaviors of the reinforced PPy membranes in aqueous environment, researchers are better guided about how to use such PPy membranes in biomedicine.

1.3.4 XPS and FTIR

Table 1.3 Surface elemental analysis by XPS (%)

	C_{1S}	N_{1S}	O_{1S}	Cl_{2P}
PPy	76.3±0.8	12.0±0.5	10.9±0.5	0.9±0.1
wPPy	75.6±2.2	12.1±1.4	12.3±1.0	-[a]

[a] undetectable

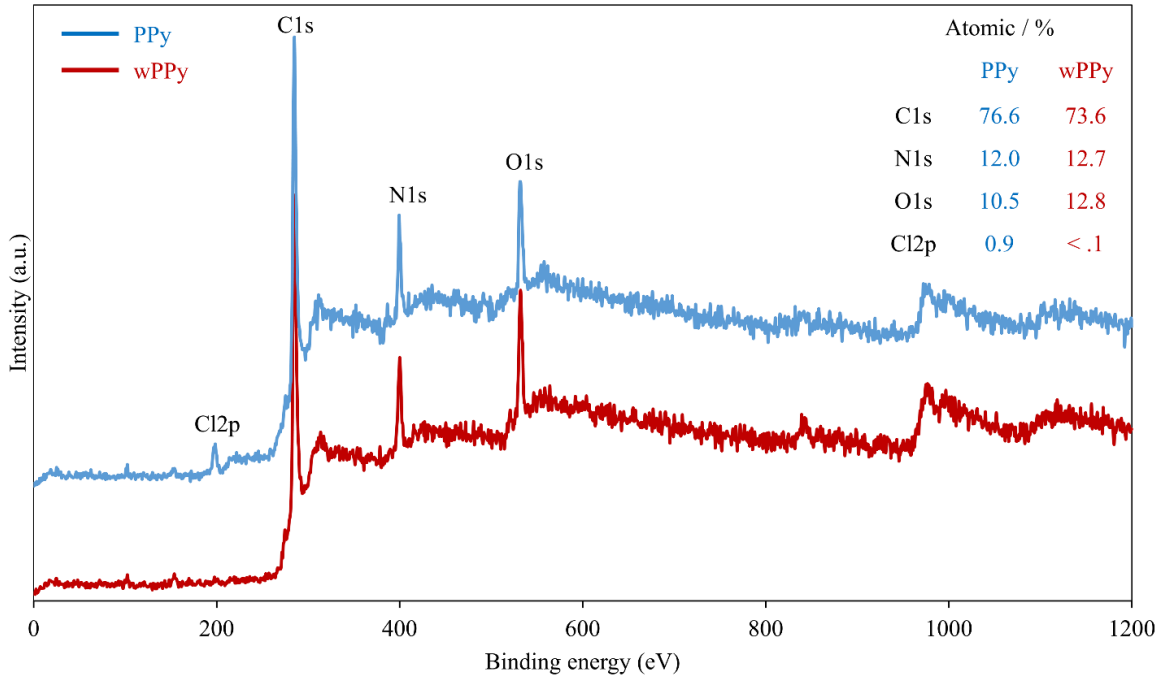

Figure 1.8 XPS survey spectrum of PPy membrane before (PPy) and after wash (wPPy).

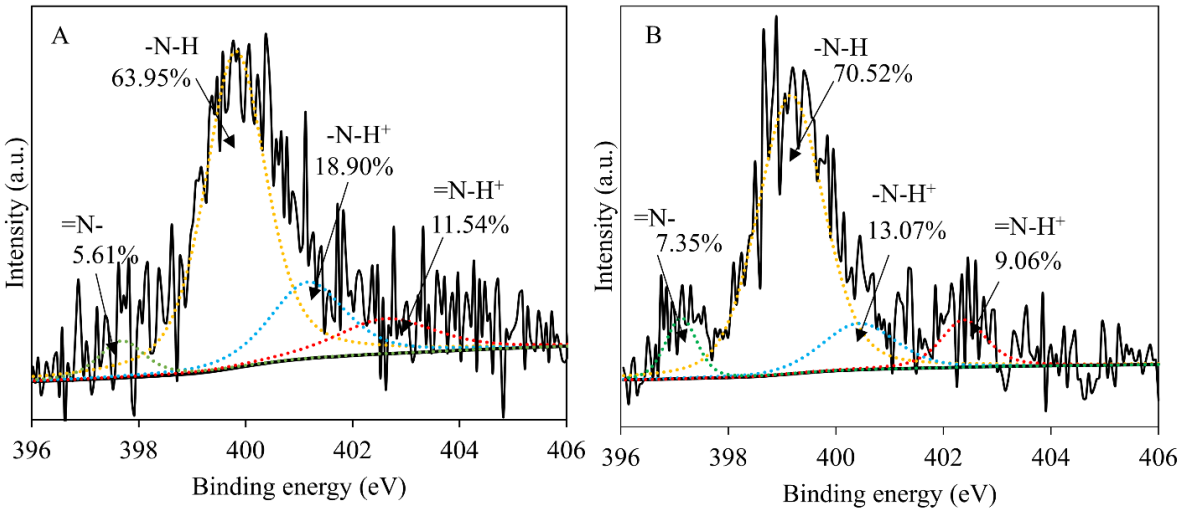

Figure 1.9 Curve fittings of the high resolution XPS spectra of N_{1s}, PPy membrane before 7 day wash (A) and after 7 day wash (B).

The influence of wash on surface elemental composition of PPy (Table 1.3) was analyzed through Student t-test. The difference in elemental composition is significant for Cl_{2p} ($p < 0.001$), but insignificant for C_{1s} ($p > 0.5$), N_{1s} ($p > 0.5$) and O_{1s} ($p > 0.05$). This means that the chemical structure of the PPy was not affected after 7 day in water except the loss of chlorine anions, as shown in Figure 1.8. The presence of a significant amount of oxygen testifies the oxidation in PPy,

which nevertheless is normal considering that the surface tested (nanotube side, with the nanotubes removed) was synthesized in water phase that inevitably contained dissolved oxygen. The loss of chlorine anions is supported by Figure 1.9, showing clearly that the amount of oxidized pyrrole rings (positively charged N) was significantly reduced from 30.44% before wash to 22.13% after wash, explaining the deterioration in conductivity before and after wash.

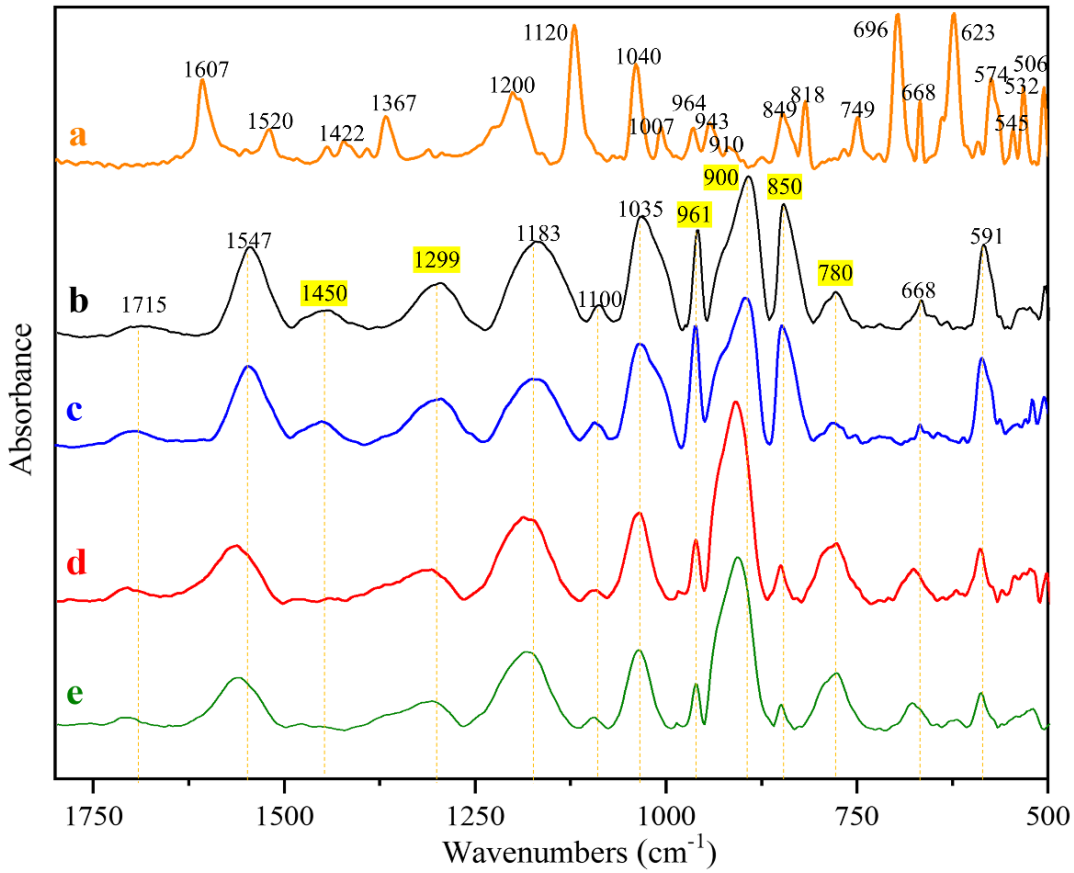

Figure 1.10 Infrared spectra of (a) MO; (b) PPy; (c) PPy-PU/PLLA; (d) wPPy; (e) wPPy-PU/PLLA.

The characteristic absorptions of the PPy and MO identified from the FTIR spectra in Figure 1.10 are summarized in Table 1.4. The MO absorptions do not exist in the spectrum of any membrane, proving that MO was completely washed out. MO is cytotoxic and its elimination is essential to insure membrane cytocompatibility. It was found that the spectra of the naked and the fiber reinforced membranes are basically identical before and after wash (Figure 1.10b vs. c, d vs. e), further proving that the electrospinning did not alter PPy chemistry. The peaks in highlight in Figure 1.10 identify the absorptions that changed before and after wash. According to literature, the absorption at 1450 cm^{-1} was due to C-N stretching in pyrrole aromatic rings [37]. The band at

1299 cm^{-1} is attributed to C-N in-plane deformation [38]. Thus after washing, pyrrole aromatic ring vibrations became weak, possibly because of the low doping ratio. After wash, the absorptions at 961 cm^{-1} (C＝C) and 850 cm^{-1} also became weaker and the ones at 900 cm^{-1} and 780 cm^{-1} became stronger. The absorption at 961 cm^{-1} is attributed to C=C-C in-plane and out-of-plane deformation, and those at 900 cm^{-1}, 850 cm^{-1} and 780 cm^{-1} are caused by C-H vibrations [38]. We conclude that the electrospinning did not bring detectable changes to PPy chemistry, and that the changes caused by washing were likely because of the dedoping and changes in oxidation states, which however requires further investigation.

Table 1.4 The characteristic absorptions of PPy and MO from FTIR spectrum
(The characteristic absorptions and assignments of the FTIR spectrum of PPy and MO)

Materials	Peak assignment	Absorptions (cm^{-1})	Ref.
PPy	Stretching of C-C and C=C	1565, 1547	[38,39]
	C-N and ring stretching vibration	1450, 1310	
	Out of plane bending of C-H and ring modes	700 to 1000	
MO	CC vibrations in the aromatic rings	1607, 1520	[40]
	CH_3 vibrations	1422	
	Azo group N=N vibration	1367	
	Sulfate groups from sulfonate species	1000 to 1200	

1.3.5 Thermal stability

As presented in Figure 1.11A&C, the thermogravimetric curves of the naked PPy show three stages of weight loss. The first stage is from 25 to 125 ℃ where the weight loss is mainly from water and low molecular weight volatiles absorbed in the membranes. In the second stage, from 125 to 300 ºC, the weight loss remains small meaning a low degradation rate, which should come from the less stable components in PPy such as the structures with oxidation. The third and most significant degradation starts around 300 ℃ and does not stop at 600 ℃, which accounts for 60% to 67% of the weight loss. The residue at 600 ℃ is 20.4 % for the original PPy and 12.6% for the PPy after the 7 day wash, indicating that when chlorine anions were used as dopant the PPy with a higher doping ratio was more stable.

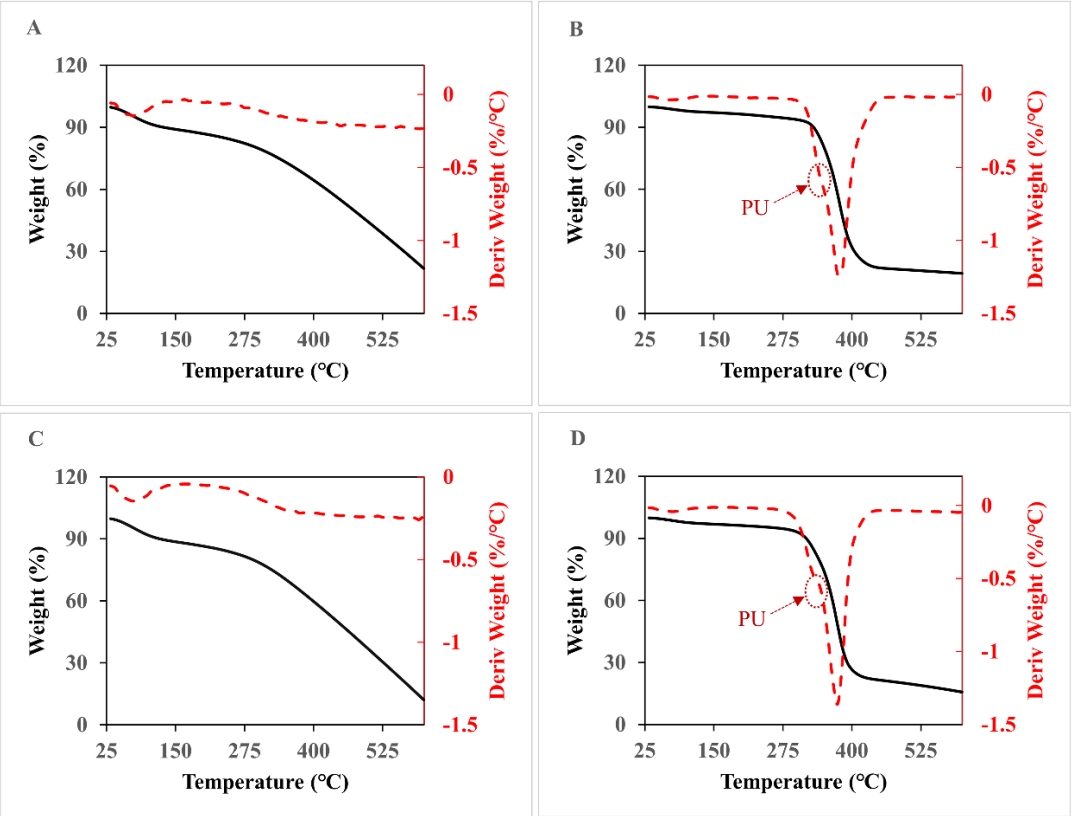

Figure 1.11 The TGA (solid black curve) and DTG (dash red curve) analyses of the membranes. (A) PPy; (B) PPy-PU/PLLA; (C) wPPy; (D) wPPy-PU/PLLA.

Table 1.5 Thermal degradation of the membranes

	Weight loss (%)				Residue (%)
	25-125 °C	**125-300 °C**	**300-600 °C**		**@600 °C**
PPy	9.4±0.4	9.6±1.7	60.7±5.0		20.4±3.0
wPPy	9.3±1.3	10.7±0.4	67.5±0.7		12.6±1.5
	25-125 °C	**125-300 °C**	**300-450 °C**	**450-600 °C**	**@600 °C**
PPy-PU/PLLA	2.5±0.2	3.7±0.1	72.6±2.3	2.5±0.2	18.7±1.9
wPPy-PU/PLLA	2.8±0.3	5.5±2.8	71.1±4.6	6.8±3.4	14.0±2.2

For the PU/PLLA reinforced PPy membranes (Figure 1.11B&D), the weight loss happened in four stages, with an initial loss of volatile maters below 125 °C, followed by a slow weight loss up to 300 °C, then a significant drop of weight from 300 to 450 °C, and finally a slow weight loss up to 600 °C. Clearly, the relatively narrow degradation temperature of PU and PLLA is largely responsible for the sharp weight loss between 300 and 450 °C. The DTG curves show a shoulder at 340 °C (arrows), which should be due to PU that is less stable than PLLA. Student *t*-test revealed

no difference ($p > 0.1$) in weight loss at the 3rd stage (72.6 *vs*. 71.1), meaning that the wash did not have impact on the thermal stability of the fibers. The significant differences at the 4th stage and in final residue are due to the de-doped and less stable PPy. To conclude, the thermal stability of PPy was affected starting from 300 °C because of the dedoping caused by the intensive wash; and the electrospinning did not affect PPy stability (Table 1.5).

1.3.6 Cytocompatibility

Figure 1.12 presents the cell proliferation after 24, 48 and 72 h of culture. It can be seen that the amount of cells on the membranes (Figure 1.12A, B and C) were comparable to that on the glass slide (Figure 1.12D, E and F). Many cells on the membrane were out of focus because of the uneven surface morphology. The quantitative data depicted by the histograms also show a similar number of cells at 24 h and 48 h. At 72 h, however, a significantly higher number of cells was found on the membranes (0.58 *vs*. 0.48, $p < 0.01$), which was probably because of the larger surface area of the PPy-PU/PLLA membrane allowing more space to proliferate. These results indicate that the reinforced membrane has an excellent cytocompatibility and can support keratinocyte growth, which indicates the potential of using such conductive membranes in skin wound care.

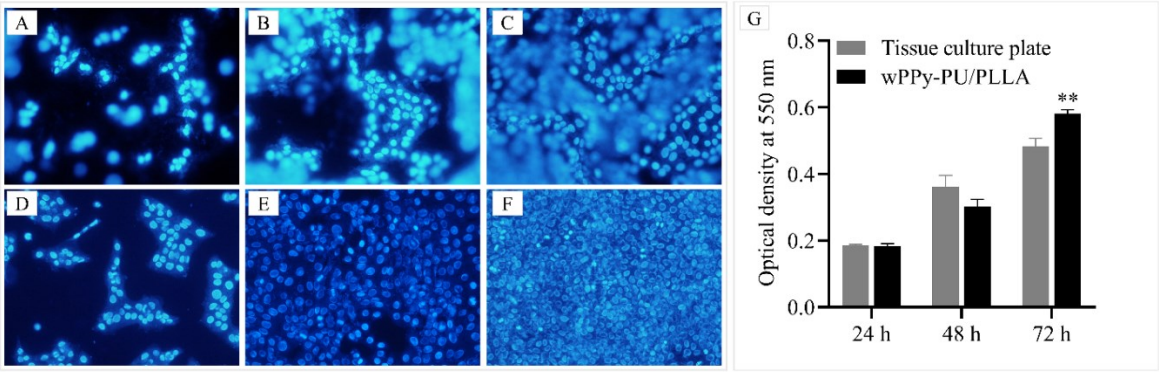

Figure 1.12 Cytocompatibility test of the PU/PLLA reinforced PPy membrane. Adhesion of human skin keratinocytes on wPPy-PU/PLLA membrane at 24 h (A), 48 h (B) and 72 h (C), showing comparable cell density to the controls on glass slide (D-F). The histograms show the proliferation of the keratinocytes, showing a comparable or higher number of cells on the wPPy-PU/PLLA membrane compared to that in the tissue culture plate. ** $p < 0.01$.

1.4. Conclusions

Electrospun PU and PLLA fibers were successfully utilized to strengthen the soft PPy membrane without sacrificing its electrical conductivity, electrical stability, thermal stability, and surface

chemistry, which for the first time makes the soft PPy membrane practically usable. The unique combination of PU and PLLA fibers ensured their strong attachment to the PPy surface, no membrane deformation in aqueous solution despite the multi-layered structure, and the sufficient mechanical strength to stand normal manipulation without damaging the membrane. The reinforced membrane remained highly flexible and light weight, and supported the proliferation of human skin keratinocytes. Such a microporous, flexible, high surface area and easy to use conductive PPy membrane is very useful for a variety of biomedical applications such as electrically stimulated cell culture and reconstruction of conductive tissues.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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In the previous chapter, a conductive PPy cell culture scaffold was successfully prepared. Considering that EF plays certain role during wounding healing, in the following chapter, the PPy scaffold was used for the electrical stimulated cell culture.

Keratinocyte plays an important role throughout skin wound healing process. Based on the literature review, only a few studies have been reported about the interactions between keratinocytes and ES. Moreover, these studies focused on keratinocyte electrotaxis. Therefore, to know more about keratinocyte behaviours under ES is valuable. This chapter explores how ES interact with keratinocytes where PPy is used as the tool to deliver electrical signals.

Chapter 2: To investigate the interaction of electrical stimulation with human skin keratinocyte

Effects of electrical stimulation on human skin keratinocyte growth and the secretion of cytokines and growth factors

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Résumé

La stimulation électrique (SE) a été largement explorée et s'est avérée efficace pour favoriser la cicatrisation des plaies. Cependant, le rôle de la SE sur les kératinocytes, un acteur majeur dans la cicatrisation des plaies, n'a pas été bien établi. L'objectif de cette étude est d'évaluer l'effet de la SE sur les comportements cellulaires et moléculaires des kératinocytes de la peau humaine. Des kératinocytes HaCaT ont été ensemencés sur une nouvelle membrane souple électriquement conductrice composée de PPy-PU/PLLA, puis exposés à différentes SE allant de 100 à 200 mV mm^{-1} pendant 6 et 24 h. Nous avons évalué l'effet de la SE sur la prolifération cellulaire, la formation de colonies, la sécrétion de cytokines et de facteurs de croissance, la production de kératines, ainsi que l'activation des kinases ERK1/2. Les résultats ont montré que les cellules stimulées électriquement présentaient une capacité proliférative plus élevée et sécrétaient plus d'IL-6, IL-1α, IL-8, GROα, FGF2, et VEGF-A. Il est intéressant de noter que la SE de 24 heures a induit une "mémoire du stimulus" en montrant une augmentation significative de l'efficacité de formation de colonies jusqu'à 5 jours post stimulation. La production de kératine 1, 5, 13 et 14 a été augmentée par la SE. Enfin, l'ES a augmenté les kinases pERK1/2. Les résultats globaux ont démontré que la SE pourrait jouer un rôle important dans la cicatrisation en augmentant la prolifération des kératinocytes et en favorisant la sécrétion de plusieurs cytokines et facteurs de croissance.

Abstract

Electrical stimulation (ES) has been widely explored and found effective in promoting wound healing. However, the role of ES on keratinocytes, a major player in wound healing, has not been well established. The present work investigated the cellular and molecular behaviors of human skin keratinocytes being exposed to ES. HaCaT keratinocytes were seeded on a novel electrically conductive and soft PPy-PU/PLLA membrane and cultured under electrical intensities of 100 or 200 mV mm^{-1} for 6 and 24 h. The factors assessed after ES include cell proliferation, colony formation, cytokines, keratins, as well as phosphorylated ERK1/2 (pERK1/2) kinases. The results showed that the electrically stimulated cells exhibited a higher proliferative ability and secreted more IL-6, IL-1α, IL-8, GROα, FGF2, and VEGF-A. Interestingly, the 24 h ES induced a "stimulus memory" by showing a significant rise in colony-forming efficiency in post-ES cells that were sub-cultured. Additionally, after stopping the 24 h ES, the productions of keratin 5 and keratin 14 were continuously increased for 3 d. The productions of keratin 10 and keratin 13 were significantly increased post the 6 h ES. Finally, the ES increased pERK1/2 kinases. The overall results demonstrated that the proliferation of keratinocytes and their secretion of cytokines and growth factors can be activated through appropriate ES to benefit skin wound healing.

2.1 Introduction

Skin is the body's first line of defense, and its integrity is important for survival. However, the capacity of skin wound healing diminishes with age and chronic diseases (*i.e.*, diabetes) [1, 2]. Indeed, skin cells around ulcer beds usually show abnormal proliferation and growth factor secretion, which compromise wound healing. Non-healing wounds such as diabetic foot ulcers represent a great challenge to patient care and a significant financial burden to the healthcare system [3]. Thus, any improvement in wound healing efficiency is welcome, not only because it can shorten hospital stay, but also because it may salvage limbs in case of non-healing ulcers.

Wound healing is a complex physiological process, which is achieved through four overlapping phases, including hemostasis, inflammation, formation of new tissue (dermis and epidermis), and tissue remodeling [4]. Each phase relies on the cross-interactions of various cell types either directly or indirectly (through growth factors and cytokines). The participating cells include platelets, neutrophils, macrophages, endothelial cells, fibroblasts, and keratinocytes. These cells are exposed or migrate to the wound site, proliferate, and secrete various mediators such as growth factors and chemokines. These mediators orchestrate cellular activities to accomplish wound healing [5]. Such cellular activities are known to respond to electrical field (EF) [6]. EF was reported to play an overriding role in guiding cell migration during wound healing [7]. Compared to normal skin wounds, diabetic skin or aged normal skin exhibits diminished EFs [8]. Inspired by this, exogenous EF has been considered an attractive tool and been explored to help wound healing. It was reported that ES could promote *in-vitro* fibroblast migration, proliferation, and differentiation [7]. As the predominant cell type in the epidermis, keratinocytes actively participate in skin wound healing [9]. During tissue formation and remodeling phases, keratinocytes proliferate and migrate (Figure 2.1) to repair the wound and restore the barrier function [10, 11]. Within the epidermis, keratinocytes are interconnected, forming different layers that constitute an efficient and a physically and chemically protective barrier [12, 13]. The basal layer consists of proliferating cells, while differentiated keratinocytes form the supra-basal layer. The keratinocytes in different layers express various keratin proteins (K) to form intermediate filaments that contribute to cell cohesion. Highly proliferative keratinocytes are located at

the basal layer of the epidermis. They express specific keratins such as K5, K14 and K19 [14], while supra-basal cells express keratins K1, K10 and K13 [15]. Keratinocytes play multiple key roles in skin repair. They are actively involved in the re-epithelialization process through proliferation, migration, and differentiation (Figure 2.1), to restore the epidermal barrier [16, 17]. During wound healing process, keratinocytes secrete different mediators (Figure 2.1), including growth factors, cytokines, and chemokines [18].

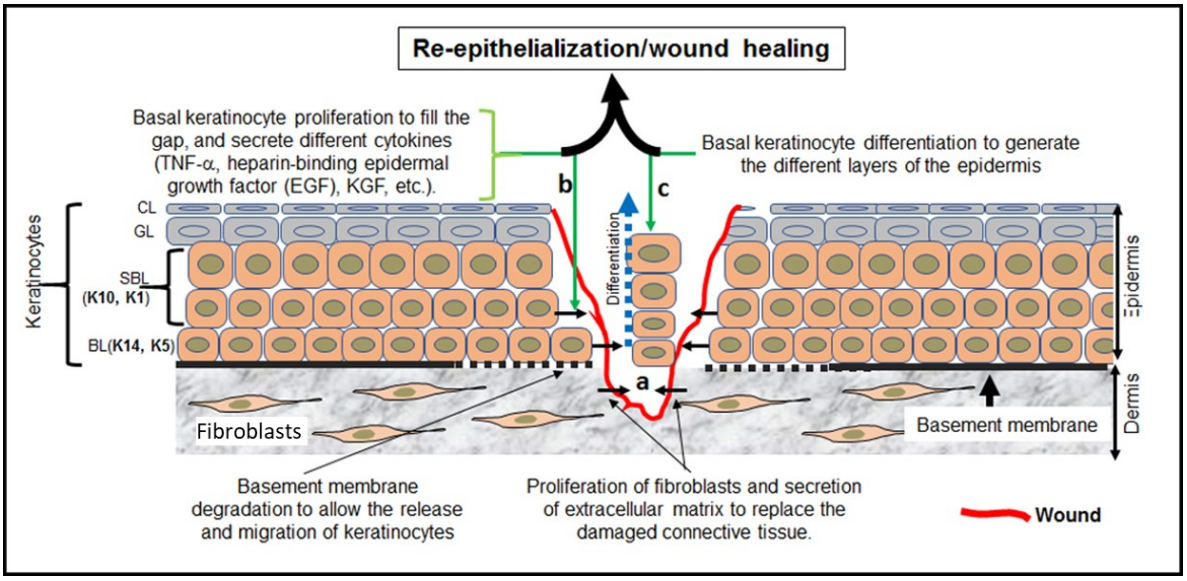

Figure 2.1 Schema summarizing the roles of keratinocytes in wound healing. Upon wound and formation of new connective tissues by fibroblasts (a), basal keratinocytes proliferate horizontally and migrate on the newly formed connective tissues to fill the wounded epidermis (b). To migrate, basal keratinocytes need to be detached from the basal membrane momentarily. Once the wound is covered horizontally, basal keratinocytes will differentiate to form the subsequent layers of the epidermis (c). Both (b) and (c) steps refer to re-epithelialization. The re-epithelialization involves multiple cytokines and growth factors secreted mostly by fibroblasts and keratinocytes near the wound. K = keratin, BL = basal layer, SBL = supra-basal layer, CL = corneum layer, and GL = granular layer.

Interestingly, ES was found to influence keratinocyte migration and proliferation [19-23]. EF was reported interacting with different types of cells through multiple signaling pathways, including epidermal growth factor (EGF) receptors, mitogen-activated protein kinase, ERK, and Src [24-26]. It has been shown that ES activated the TGFβ1/ERK/NF-κB signaling pathway in primary human skin fibroblasts [27] and ERK1/2 and the p38 mitogen activated protein (MAP) kinase pathways in primary human skin keratinocytes [21]. The culture of primary human keratinocytes needs the use of feeder cells (irradiated 3T3 fibroblasts) to promote

keratinocytes growth and prevent their differentiation [28]. These feeder cells may have effect on keratinocytes when exposed to ES or other stimuli. This limitation could be solved by using HaCaT cell line.

HaCaT cell is a spontaneously immortalized nontumorigenic keratinocyte cell line generated from adult human skin. This cell line offers several advantages compared to primary human keratinocytes [29]. HaCaT cells can be maintained for a long-term growth without the use of feeder cells or specific growth factors, compared to primary human keratinocytes. HaCaT cells exhibit both the fundamental functions of the primary human keratinocytes and the stable plating efficiency, *i.e.*, steadily expressing specific proliferation (Keratins 5 and 14) and differentiation markers (Keratins 1 and 10) [30]. This work, therefore, used HaCaT cells to evaluate the effect of ES on keratinocyte behaviors.

ES can be delivered through conductive polymers (CPs) [31]. Owning to the unique electrical conductivity and biocompatibility, CPs provide an attractive option to deliver ES to cells or tissues. CPs can support cell grows on them, and at the same time electrically stimulate the cells with the EF applied to the CPs. This is an easy approach and can avoid the electrochemical reactions when using electrodes. Polypyrrole (PPy) has received a great interest in the field of ES and skin wound healing [32], as it exhibits the good electrical properties, excellent biocompatibility, and ease of synthesis. These properties endow PPy with the great potential and advantages in tissue engineering and regenerative medicine applications. However, PPy has some limitations because it is brittle, insoluble, and infusible. To solve such limitations, PPy can be mixed with polymers having sufficient rigidity, strength and ductility, *i.e.*, poly(L-lactide) (PLLA), polycaprolactone (PCL), polyurethane (PU), etc., to form a PPy-filled composite membrane [33]; or be coated on the polymeric textiles, *i.e.*, a polyethylene terephthalate (PET) fabric, to produce a PPy-coated conductive fabric [34]. These products as ES vehicles have been successfully used and showed positive outcomes. However, for the composite membrane, the supporting polymer sacrifices the conductivity significantly; for the surface coating, although the thin PPy layer can be as conductive as the pure PPy, the conductivity deteriorates rapidly once used in an aqueous environment due to dedoping. In this work, therefor, a novel soft PPy-PU/PLLA membrane was used. This membrane maintained the conductivity of a pure PPy membrane and at same time achieved an enhanced mechanical strength, making it a practical tool to deliver ES.

2.2 Methods

2.2.1 Soft PPy-PU/PLLA membrane and ES cell culture device

The pure PPy membranes were synthesized through a template-assisted interfacial polymerization technique [35]. Briefly, pyrrole monomers (Sigma-Aldrich, St Louis, MO, USA) were distilled and then dissolved in chloroform (reagent grade, Fisher Chemicals, Ottawa, Canada) to form solution A. Ferric chloride (Sigma Aldrich) as the oxidant and methyl orange (Sigma-Aldrich) as the template were dissolved in deionized water to form solution B. Solution A was firstly added into a beaker, on top of which solution B was slowly added. The beaker was kept at -4 ºC till a PPy membrane was formed at the chloroform/water interface. The membrane was carefully collected and thoroughly washed to remove impurities. After drying, a circular soft PPy membrane about 15 cm in diameter and 200 □m in thickness was obtained. The thickness was measured under 19.76 kPa using a MTG-DX2 digital gauge (Rex Gauge Company, Buffalo Grove, IL, USA).

To increase the handling property of the fragile PPy membrane, PU Tecoflex 80A (Lubrizol, Avon Lake, OH, USA) and PLLA (Hycail, Turku, Finland) fibres were sequentially electrospun onto the bubble side of the PPy membrane [36] as described below.

The PU and PLLA solutions of 15 wt% were separately prepared by adding solid polymer pellets to $CHCl_3$, and then stirred at room temperature overnight. A volume of 1.5 mL of the polymer solution was then fed into a syringe that was then loaded to a handheld electrospinning device. Fibres were spun through a 25-gauge needle to cover 241 cm^2 of PPy surface (*i.e.*, 0.93 mg cm^{-2}) at a feeding rate of about 2.25 mL h^{-1} under a directed current (DC) voltage about 9 kV at 25 °C and 65% humidity. The bubble side of the PPy membranes was used as the collector, which was 25 cm away from the needle. The PU fibers were firstly spun, followed by the PLLA fibers. The fiber reinforced PPy membrane was then placed in an oven at 40 °C overnight to evaporate $CHCl_3$.[36] The membranes were thoroughly washed, dried, and then cut into specimens of 7.0 × 2.5 cm^2 in size. These specimens were assembled at the bottom of our home-made multi-well ES cell culture plates, then sterilized with ethylene oxide gas (Anprolene AN2000, Anderson products Inc., Haw Drive, NC, USA) at room temperature before use. The two ends of the rectangular membrane specimens extended

to the outside of the culture wells were connected to a waveform generator and a real-time monitoring device (Keithley 2700, Cleveland, OH, USA).

2.2.2 Keratinocyte culture

HaCaT cells (Cedarlane CELLutions Biosystems, Burlington, Canada) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum in 75 cm^2 flasks in a humidified incubator at 37 °C with 5% CO_2. Once the cultures reached 80% confluence, the cells were detached from the flasks using 0.05% trypsin and used to investigate the effect of ES, as summarized in the schema below (Figure 2.2). Cell culture medium was changed every 2 d.

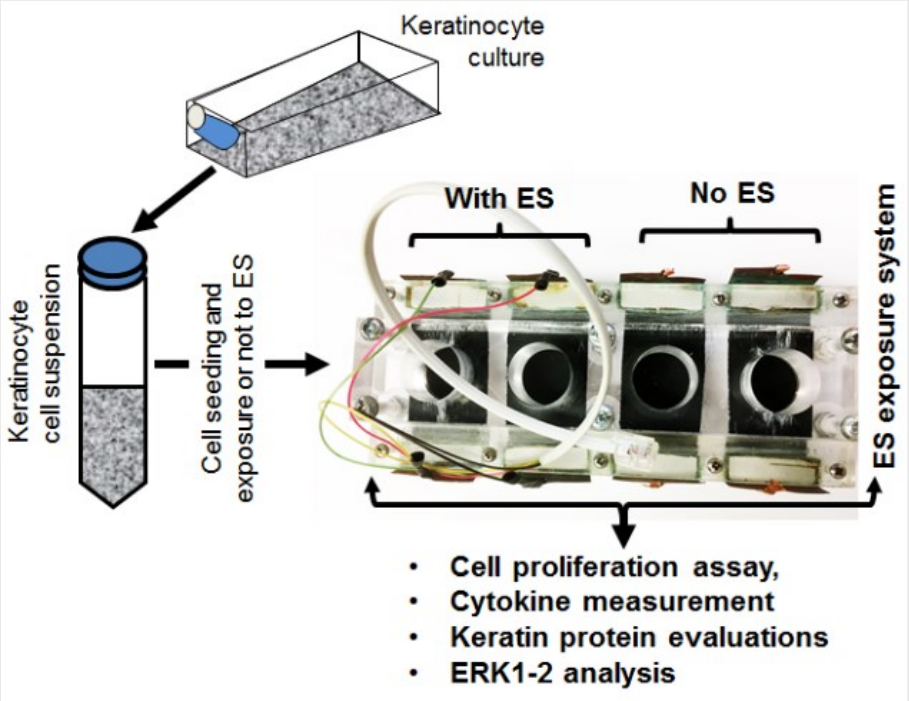

Figure 2.2 Schematic diagram of the ES set up. The PPy-PLLA/PU membranes (7 cm × 2.5 cm each) were fixed in a 4-well cell culture plate (between the cell culture well and the base plate) using screws, with two ends out of the cell culture well to connect with the electrical circuit.

2.2.3 HaCaT cell viability and growth following ES

Prior to cell seeding, the membranes in the ES cell culture plates were incubated firstly in PBS for a day and then in DMEM overnight. Thereafter, 3 ml of HaCaT cell suspension was seeded onto the membrane at a density of 5×10^4 cells cm^2. After 24 h culturing, the media

were refreshed, and the PPy-PU/PLLA membranes were supplied with a constant DC potential of either 100 or 200 mV mm^{-1} for 6 and 24 h. In such a way, the keratinocytes on the surface of the membranes were exposed to the specific EF. The selected ES intensities were shown to be non-toxic to human skin fibroblasts [37]. They were within the physiological range (40–180 mV mm^{-1}) of endogenous EF [24, 38]. After exposure to ES, the cells were further cultured for 24 h prior to analysis. Control groups followed the same conditions without exposure to ES. Cell viability was measured using the MTS (methyl thiazolyl tetrazolium salt) colorimetric assay (ab197010, Abcam, Cambridge, United Kingdom). Each experiment was repeated minimally three times.

2.2.4 Lactate dehydrogenase (LDH) activity

Following HaCaT cell exposure to ES and culture for an additional 24 h, the supernatants were collected, centrifuged to remove debris, then subjected to a LDH cytotoxicity assay (Promega, Madison, WI, USA). Briefly, 50 µl of each culture medium were transferred to a flat-bottom 96-well plate in the presence of 50 µl of reconstituted substrate mix. The mixture was incubated in the dark at room temperature for 30 min. To stop the reaction, an acid solution (50 µl) was added to the well. The absorbance of the wells was read at 490 nm with an X-Mark microplate spectrophotometer (Bio-Rad, Mississauga, ON, Canada). As a positive control, keratinocytes were incubated in the presence of 1% Triton X-100 to induce 100% cell death. Negative control was obtained by culturing keratinocytes under normal cell growth conditions.

2.2.5 ES effect on colony formation

Colony-forming efficiency (CFE) was used to study the growth potential of the ES-treated HaCaT cells [39]. Briefly, 24 h after ES, HaCaT cells were detached from the PPy-PU/PLLA membrane using 0.25% trypsin, and 3×10^4 cells collected from each ES condition were seeded in a 6 well tissue culture plate. After 6-day of incubation at 37 °C in 5% CO_2 and with the medium refreshed every 48 h, the cultures were fixed in paraformaldehyde and stained with crystal violet. The colonies with five cells and more were counted under an optical microscope. Control groups without ES were cultured and treated following the same procedure. CFE was expressed as a percentage of the counted colonies to the total number of

plated cells. The results were presented in the form of a comparison between the control and ES groups, with the control group being considered 100%.

2.2.6 Release of IL-1α, IL-6, IL-8, GROα, FGF2, and VEGF-A

After ES and the additional 24 h culture, cell culture supernatants were collected, filtered through 0.22 μm filters, and then used for cytokine array analysis. The cytokines, chemokines, and growth factors in the supernatants were detected using a MILLIPLEX Human Cytokine/Chemokine 71 plex kit (Millipore, St. Charles, MO, USA). The multiplexing analysis was performed using the Luminex™ 100 system (Eve Technologies Corp., Calgary, Canada). According to the manufacturer, assay sensitivities range from 0.1 to 9.5 pg mL^{-1}. Each experiment was repeated four times and the means ± SD were calculated.

2.2.7 Western blot analysis of keratin production

HaCaT cells (5×10^5 cells cm^2) were cultured, electrically stimulated, and further cultured for 24 h as previously described. When finish, cells were trypsinized from the PPy-PU/PLLA membrane and re-cultured in a flask. After 60 h culture, cells were collected from the flask and lysed in a lysis buffer (Sigma-Aldrich) supplemented with an anti-phosphatase cocktail III (Sigma-Aldrich). Concentrations of the extracted proteins were quantified through the Bradford assay. Equal amounts of total protein (20 - 40 μg) in a reducing buffer (61.5 mM Tris, 100 mM dithiothreitol (DTT), 2% lauryl sodium sulfate (SDS), 10% glycerol) were boiled for 5 min and separated through SDS-polyacrylamide gel electrophoresis using a 10% polyacrylamide gel. The proteins were then transferred to the PVDF membranes using a refrigerated Tris–glycine transfer buffer (25 mM Tris, 192 mM glycine, 100 μM Na_3VO_4, and 20% methanol) for 1 h at 100 V. The PVDF membranes were incubated overnight with the primary antibodies against K5 (ab52635, Abcam), K14 (ab7800, Abcam), K10 (ab76318, Abcam), and K13 (sc-390982, Santa Cruz, CA, USA). The membranes were then washed and incubated for 1 h with the appropriate peroxidase-conjugated secondary antibodies. Detection was performed using the VersaDoc 5000MP Imaging System (Bio-Rad, Hercules, CA, USA), and photographs were taken and analyzed with the Quantity One software (Bio-Rad, Hercules, CA, USA).

2.2.8 MAP kinase signalling pathways

To assess the contribution of MAP kinases to the ES-mediated HaCaT growth, cells were cultured for 24 h on the PPy-PU/PLLA membrane, then preincubated for 45 min with and without 10 μM of inhibitors to ERK (PD98059, InvivoGen, San Diego, CA, USA) prior to ES exposure for 6 or 24h. After the ES exposure regime, total proteins were extracted and separated through SDS-polyacrylamide gel electrophoresis using a 12% polyacrylamide gel as described above. The proteins were subjected to Western blot analyses using both total (4377S, NEB, MA, USA) and phosph-ERK1/2 antibodies (4695S, NEB).

2.2.9 Statistical analysis

Data was presented as mean ± standard deviation (SD) of at least three experiments. The statistical significance of the differences between the control and the test values was determined by one-way analysis of variance. Posteriori comparisons were performed using Tukey's method. *P* values were declared significant at < 0.05. The data were analyzed using the SPSS (IBM SPSS Statistics 19) and GraphPad Prism 8 (GraphPad Software).

2.3 Results

2.3.1 ES promoted HaCaT viability and proliferation

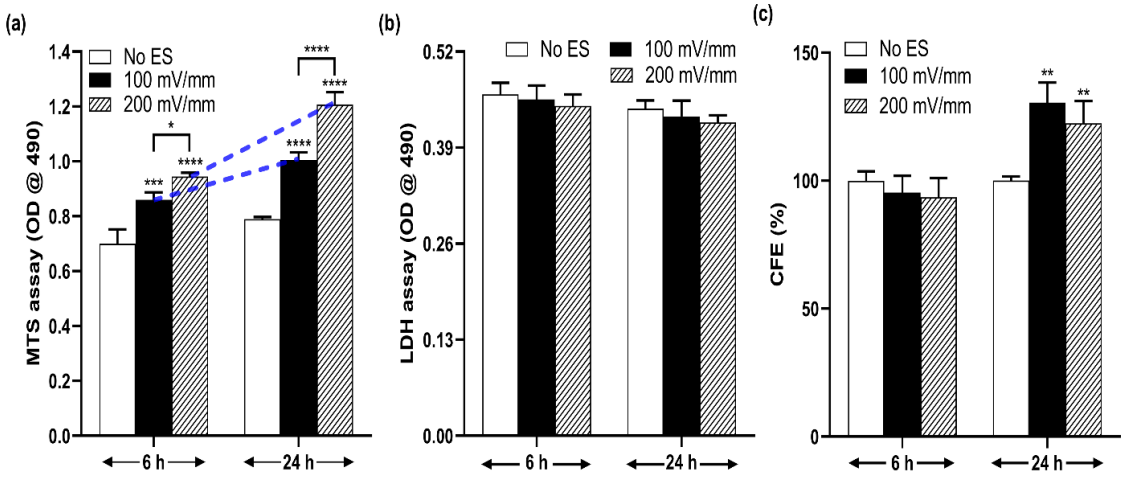

Figure 2.3 Electrical stimulation (ES) through PPy-PU/PLLA membrane promoted HaCaT viability/growth and the long-term growth and colony formation. Cells were seeded onto the conductive PPy-PU/PLLA membrane for 24 h and subjected to a 100 or 200 mV mm^{-1} ES for 6 and 24 h. (a) Cell viability and proliferation were measured by MTS (n = 4), (b) Cell

cytotoxicity was measured by the LDH (n = 4) assays, (c) the electrically stimulated cells were collected and plated in 6 well plate and cultured for 6 d, the colonies were counted under an optical microscope to calculate CFE (n = 8). Data are presented as means ± SD. Significance was determined by comparing the control values with those obtained after ES. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

Keratinocytes were cultured onto a conductive PPy-PU/PLLA membrane that was produced and characterized previously [36]. The viability/proliferation of HaCaT was significantly promoted by the ES at either 100 or 200 mV mm^{-1} for both 6 and 24 h, in comparison with the controls (No ES), as shown in Figure 2.3(a). Moreover, cell viability/proliferation increased with the increase in ES intensity (from 100 to 200 mV mm^{-1}). Besides, a similar tendency was also observed when the ES exposure increased from 6 to 24 h. It also appears that the exposure to 200 mV mm^{-1} for 24 h was more effective on keratinocyte growth, as indicated by the steeper slops of the dash lines in both Figure 2.3. These results indicate that all four combinations of the ES parameters could accelerate the proliferation of HaCaT cells. In addition, the cell cytotoxicity was measured using the LDH assay, as shown in Figure 2.3(b). It shows no significant effect of ES on LDH activity, either in intensity or in duration.

2.3.2 ES promoted the formation of HaCaT cells colonies

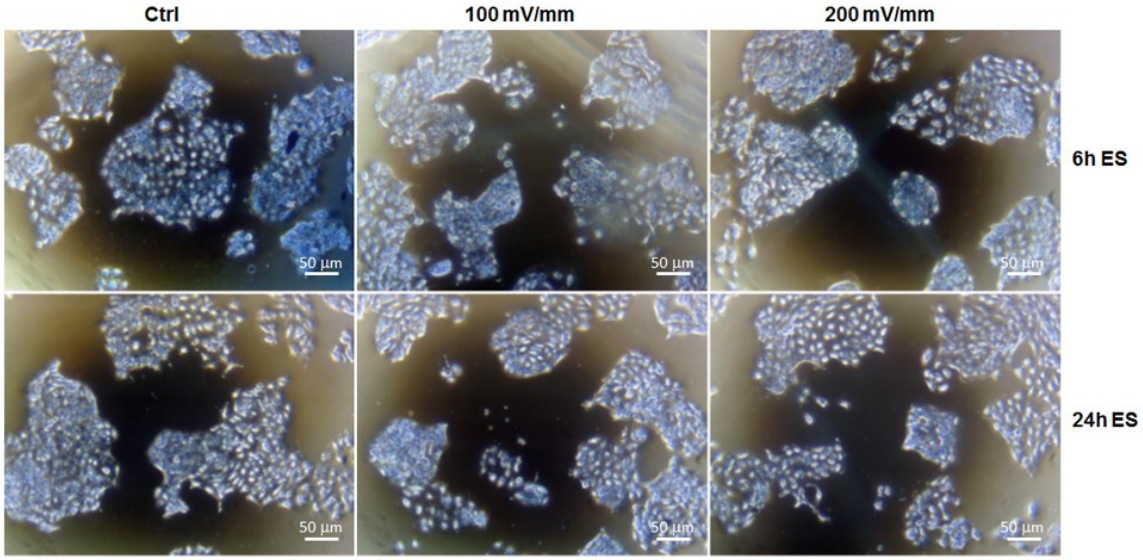

Figure 2.4 ES has no adverse effect on keratinocyte morphology. Keratinocytes were exposed to ES for 6 or 24 h. They were then detached from the conductive membrane, seeded in tissue culture plate, and cultured for 6 d. The cells were then stained with crystal violet, observed under an optical microscope, and photographed. Representative photos, *n* = 4.

The long-term effect of ES on HaCaT cells is also a critical indicator, which was investigated by observing the clone formation of the ES-exposed HaCaT cells for 6-day post-ES (Figure 2.3(c)). There is no significant difference in CFE between the 100 and 200 mV mm^{-1} ES for both 6 and 24 h exposures. There is also no significant difference between the control and the ES groups when the stimulation was 6 h. However, the CFE of the ES groups at both intensities displayed a significant ($p \leq 0.01$) increase with respect to the control groups when the duration of ES reached 24 h (Figure 2.3(c)). The cell morphology of the cells after exposure to ES for 6 and 24 h was comparable to those non-exposed cells. Indeed, keratinocytes showed small size with a cuboidal form and small nucleus and cytoplasm volumes (Figure 2.4).

2.3.3 Effect on the levels of IL-6, IL-8, IL-1α, GROα, FGF2, and VEGF-A

As shown in Figure 2.5(a), the interleukin 6 (IL-6) secretion increased significantly under all four ES conditions than the controls. In particular, the increase was about 12%-14% of the controls after 6 h ES, and about 20% to 24% after 24 h ES. It was also noted that the two ES intensities did not cause any significant difference. Finally, the levels of IL-6 in all 24 h groups, including control are significantly higher than that in the 6 h groups.

Figure 2.5(b) shows significantly higher IL-8 levels in the four ES groups except in the 6 hr/200 mV mm^{-1} group, as compared to the controls. All other three ES groups recorded a 24%-28% increase vs. controls. Interestingly, at 200 mV mm^{-1}, the secretion of IL-8 showed a latent period, with a slight but non-significant increase following the 6 h ES.

Figure 2.5(c) shows significant increases in IL-1α in all ES groups compared to the controls. Moreover, the variations caused by different ES intensities and stimulation periods are also significant. Specifically, the level of IL-1α increased 30% after 6 h at 100 mV mm^{-1}, 51% after 6 h at 200 mV mm^{-1}, 35% after 24 h at 100 mV mm^{-1}, and 10% after 24 h at 200 mV mm^{-1}. The secretion of IL-1□ increased with the increase in ES intensity when the stimulation time was 6 h. For a longer stimulation time, the secretion was lower at high intensity.

There was a significant increase in the level of GROα for all ES groups (Figure 2.5(d)). The percent increases are 86% and 121% for the 6 h stimulation at 100 and 200 mV mm^{-1},

respectively. For the 24 h stimulation, the increases are 91% at 100 mV mm^{-1} and 106% at 200 mV mm^{-1}. Again, for the short time stimulation, the two ES intensities resulted in a significant difference.

Figure 2.5(e) shows that the secretion of FGF2 was significantly increased in all ES groups vs. controls, ranging from 31% to 73%. The significant increase of FGF2 by ES was obtained at 6 h, while at 24 h there was no significant difference compared with the control.

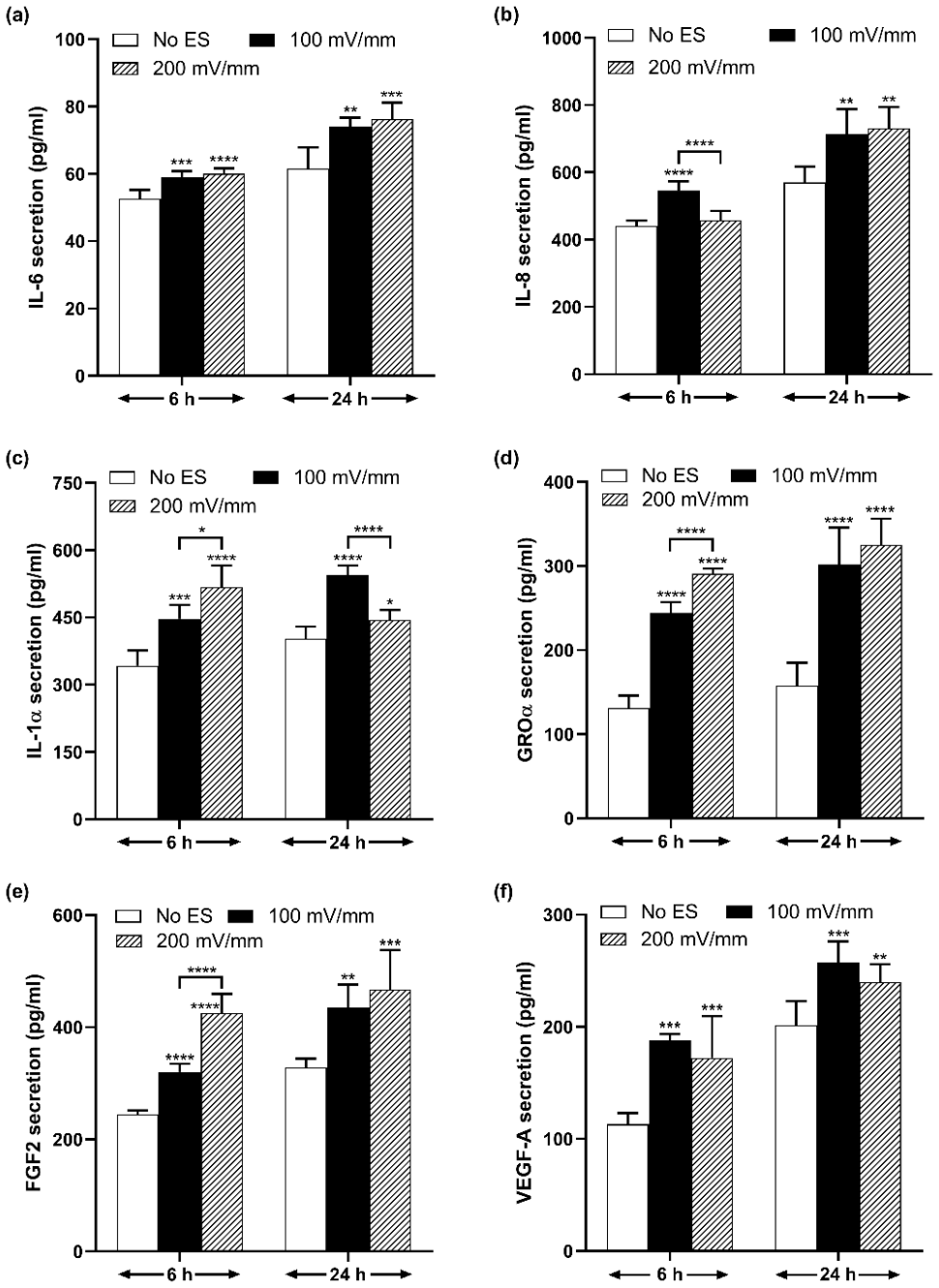

Figure 2.5 ES increased the secretion of (a) epidermal IL-6, (b) interleukin 8 (IL-8), (c) interleukin 1 alpha (IL-1α), (d) chemokine (C-X-C motif) ligand 1 (CXCL1 or GROα), (e) epidermal basic fibroblast growth factor (FGF2), and (f) vascular endothelial growth factor A (VEGF-A) by HaCaT. After the exposure of the cells to ES at 100 or 200 mV mm^{-1} for 6 and 24 h, the supernatants were collected, and the IL-1α, IL-6, IL-8, GROα, FGF2, VEGF-A levels were measured by cytokine array assay. Significance was determined by comparing the control values with those obtained after ES, n = 4. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

The increase in VEGF-A is shown in Figure 2.5(f). ES significantly upregulated the level of VEGF-A in all stimulated groups. Compared to the 24 h stimulation, the 6 h stimulation demonstrated a higher efficiency, resulting in a 66% increase at 100 mV mm^{-1} and a 52% increase at 200 mV mm^{-1}. The corresponding values for the 24 h stimulation are 28% and 19%. The two ES intensities did not cause any significant difference.

2.3.4 Effect on keratin production

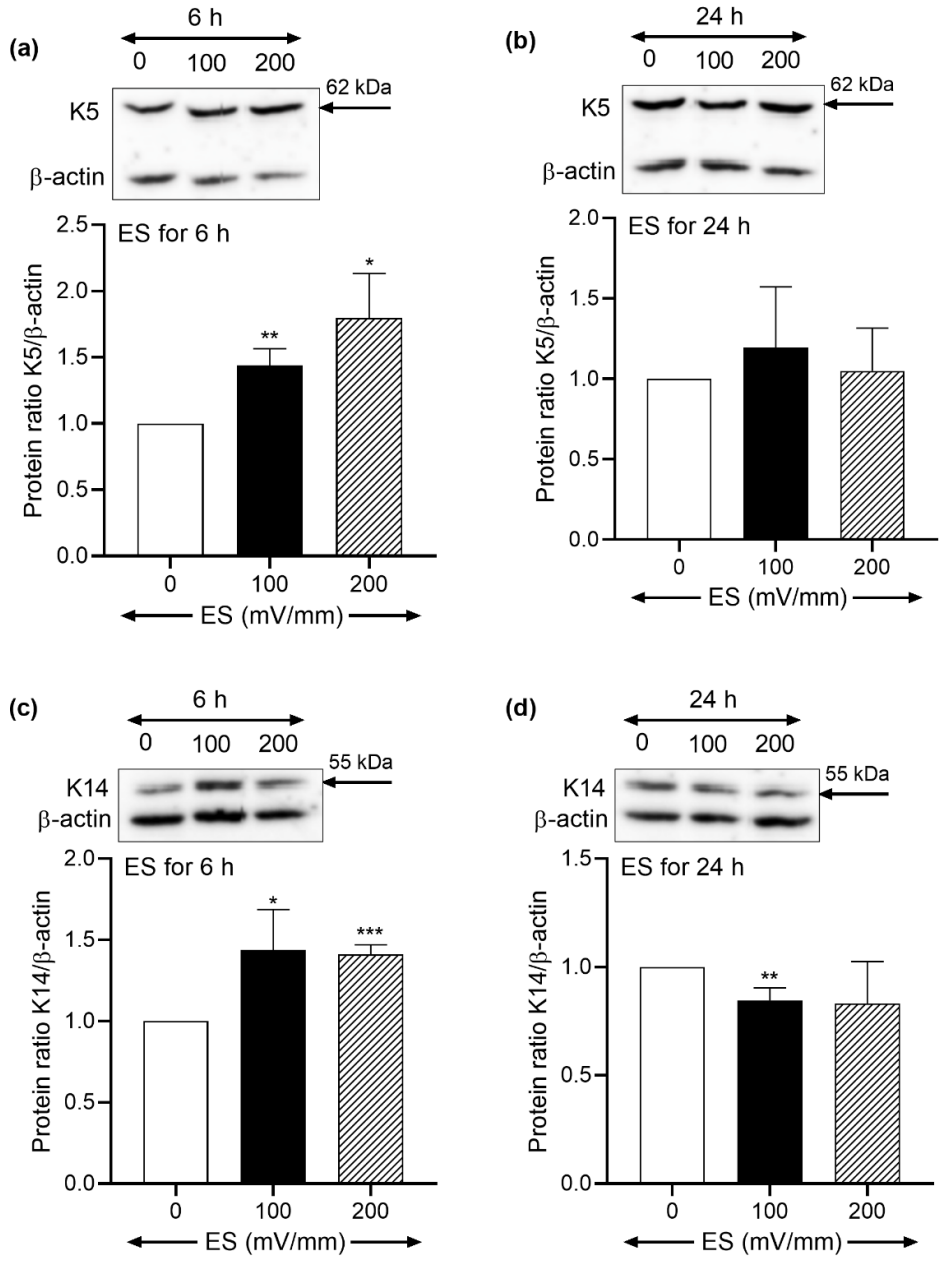

Figure 2.6 A 6 h ES increased the production of keratin 5 (K5) and keratin 14 (K14) by HaCaTs. Cells were exposed/not exposed to 100 or 200 mV mm^{-1} for 6 and 24 h. K5 and K14 protein productions were evaluated by Western blot. The values of the scanned bands are presented. Significance was determined by comparing the control values and those obtained after ES, $n = 4$. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

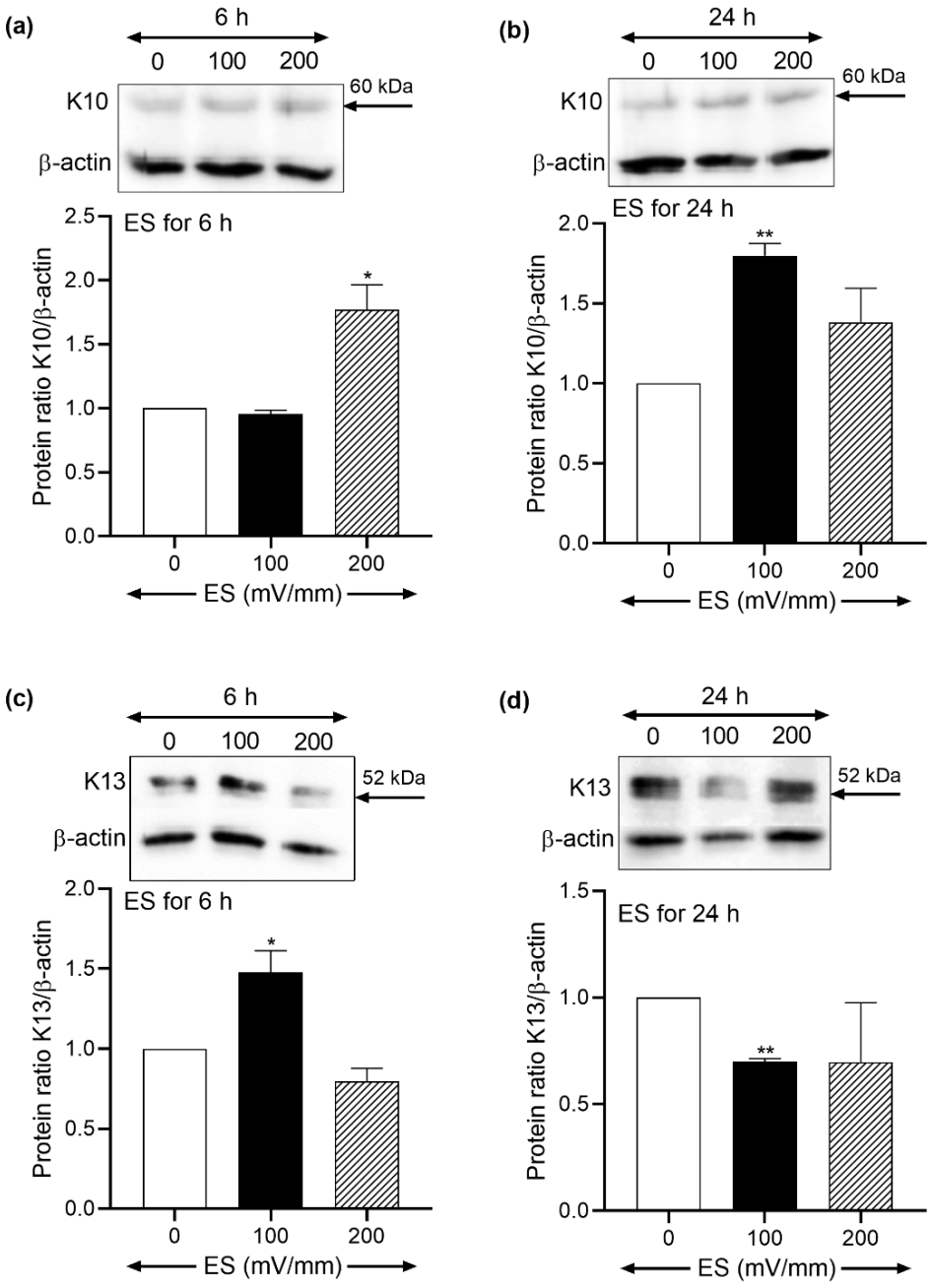

Figure 2.7 ES differentially stimulated the production of keratin 10 (K10) and keratin 13 (K13) by HaCaTs. Cells were exposed/not exposed to 100 or 200 mV mm^{-1} for 6 and 24 h. K10 and K13 protein productions were evaluated by Western blot. The values of the scanned bands are presented. Significance was determined by comparing the control values and those obtained after ES, $n = 4$. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

The production of K5, K10, K13 and K14 with respect to β-actin was shown in Figure 2.6 and Figure 2.7. It can be seen from Figure 2.6(a) that the 6 h ES significantly increased the

secretion of K5 and K14 by about 50%. The difference in ES intensity did not have any impact on the other hand. However, for the 24 h stimulation, there is little effect on Keratin 5 (K5) production. In fact, slight decreases in K14 were found after 24 h ES (Figure 2.6(d)).

Figure 2.7(a) and (c) showed that both K10 and K13 productions were significantly increased when the keratinocytes were exposed to a 6 h ES, but at different intensities. The increase in K10 was obtained with 200 mV mm^{-1} ES; but the increase in K13 was obtained with 100 mV mm^{-1} ES. For the 24 h ES, as showed by Figure 2.5(b) and (d), the production of K10 was significantly increased with 100 mV mm^{-1} ES, while there was a significant decline in K13 production after the 100 mV mm^{-1} ES.

2.3.5 Signalling molecules

As shown in Figure 2.8, the exposure to ES for 6 h increased phosphorylated ERK1/2 (pERK1/2) production compared to the controls. The pERK1/2 increase was greater at 200 mV mm^{-1} as compared to the 100 mV mm^{-1}. Meanwhile, the presence of the ERK1/2 inhibitor effectively inhibited the production of pERK1/2 at both ES intensities. This suggests that the effect of ES on keratinocytes involves pERK1/2 MAP kinase signaling pathway.

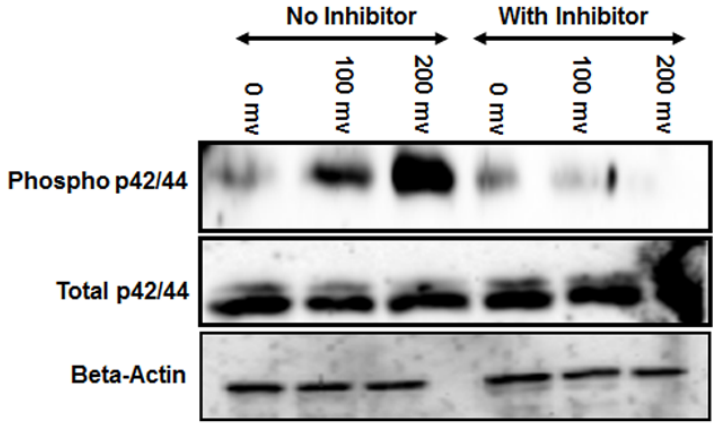

Figure 2.8 Production of the phosphorylated ERK1/2 (pERK1/2) in keratinocytes exposed to electrical stimulation. Cells were pre-incubated or not with ERK inhibitor (PD98059) for 45 min, then exposed or not to 100 or 200 mV mm^{-1} for 6 h. Proteins were collected to analyze total and pERK1/2 by Western blot.

2.4 Discussion

It is well known that keratinocytes have a pivotal role in re-epithelialization during the wound healing process. This involves keratinocyte migration and proliferation to cover the wound bed [4]. Multiple stimuli such as EF were reported to promote keratinocyte migration contributing to wound healing [22]. This is supported by our study showing that ES at 100 and 200 mV mm^{-1} was cytocompatible promoting the proliferation of HaCaT cells. The proliferation of keratinocytes goes through the formation of colonies [40]. Colony-formation is a well-recognized indicator of the number of cells having relatively high proliferative potential and reflects the proliferative potential of a single cell under a given set of culture conditions. This study demonstrated that the ES promoted the formation of keratinocyte colonies. Compared with controls, the high adhesion/growth activities were maintained for 6-day for the cells that were stimulated for 24 h at either 100 or 200 mV mm^{-1}. However, for those only stimulated for 6 h, such acquired extra cell proliferation/adhesion abilities disappeared in the 6-day colony formation experiment. These results might indicate that the "growth memory" in HaCaT cells can be established after 24 h ES. Further studies are needed to demonstrate whether keratinocytes would keep such a growth memory with a longer ES time. The famous experiments that Ivan Pavlov conducted with his dogs well demonstrated that behaviors established by an external stimulus could be memorized in an animal [41]. And it was reported that human memory was enhanced after ES to brain cells [42]. So, this study opens a new scene of ES effects on skin wound healing. Indeed, it would be interesting to evaluate the effect of ES on HaCat cell migration after scratch (an *in vitro* wound healing model). However, with our non-transparent conductive PPy-PU/PLLA membranes, we cannot perform such an experiment for the moment.

In keratinocytes, different cytoskeleton proteins such as keratins play significant roles in epidermis mechanical integrity. They promote and maintain cell proliferation [14, 43]. Among the numerous keratins, K5 and K14 are strongly expressed by highly proliferative keratinocytes, and their production is downregulated as cells differentiate. Because we demonstrated that exposure to ES increased the production of K5 and K14, this supported the idea that ES can contribute to keratinocyte proliferation and by doing so can play a significant role in the wound healing process as previously suggested [21]. Skin keratinocytes are also

producers of other keratins such as K1, K4, K10, K13. These proteins are more expressed in differentiated keratinocytes and are major constituents of the intermediate filament cytoskeleton in supra-basal epidermis [44-47]. Because we demonstrated that ES increased the production of K10 and K13, this may suggest the implication of ES in keratinocyte differentiation contributing to the formation of epidermal cell layers [48].

As keratinocytes are the first to encounter external stimuli when skin injury happens, they initiate the immune response by increasing the production of various inflammatory mediators [49]. Keratinocytes are the source of multiple mediators, including IL-1α, IL-8, IL-6 and GROα that are the key cytokines implicated in keratinocyte immune responses. Among them, IL-1α is a key pro-inflammatory mediator that can mediate keratinocyte proliferation by stimulating fibroblasts to secrete cytokines inducing keratinocyte division [50]. IL-6 was reported to promote inflammatory response during the inflammation phase but induce fibroblast migration and keratinocyte proliferation and migration in the proliferation phase [51]. IL-8 not only promotes inflammation but also plays an anti-inflammatory role during wound healing. Besides, IL-8 is a mitogen for keratinocytes and supports angiogenesis [52, 53]. Like IL-8, it has been shown that GROα also contributes to wound re-epithelization [54]. Overall, we demonstrated that the ES protocols used in this work could upregulate the secretion of IL-1α, IL-6, IL-8, and GROα. As these cytokines were reported to play active roles in keratinocyte proliferation and differentiation, which was also supported by the colony formation experiment in this work, these ES protocols may be used to accelerate tissue re-epithelialization.

Keratinocytes also secrete a variety of growth factors, including FGF2 and VEGF-A. These factors play crucial roles throughout the wound healing process [55]. We demonstrated that ES increased the secretion of FGF2 and VEGF-A by keratinocytes. FGF2 and VEGF-A are essential to support cell growth and wound healing. FGF2 activates fibroblast migration, while VEGF-A plays an important pro-angiogenic role [56, 57]. Consequently, the increase in FGF2 and VEGF-A reported in this study suggests the active role of ES in wound healing.

Given the above findings, we investigated the signaling pathway that may link the ES with the observed cellular activities. ERK1/2 has been well known for its crucial role in regulating cell cycle, proliferation, and development [58, 59]. From the increased production level of the

phosphorylated ERK1/2 after ES, it is reasonable to suggest that the ES activated the ERK1/2 pathway leading to the activation of the keratinocytes and the production of different keratins and mediators. This, however, does not exclude the possibility that other signaling pathways may also have been activated by ES.

Finally, it is worth pointing out that ES as a tool can differentially regulate cellular activities. It not only can upregulate but also can down-regulate the secretion of important proteins. For example, K10 was upregulated by 24 ES at both intensities, but K13 was down-regulated by the same conditions. This is about differential regulation. Further study is required to explore this line of inquiry.

2.5 Conclusions

This study, for the first time, explored the effect of ES on HaCaT keratinocytes through a flexible electrically conductive PPy-PU/PLLA membrane. It revealed the immediate effect on the secretion of key mediators of inflammation and wound healing and the long-term benefit in promoting keratinocyte colony formation. We also demonstrated that keratinocytes differentially reacted to the particular combinations of the ES parameters. Finally, we revealed that the ERK1/2 signaling pathway was involved in the electrical activation of the keratinocytes. This work, therefore, demonstrated that the ES protocols used in this study may contribute to tissue re-epithelialization and accelerate wound healing.

Data availability statement

All data that support the findings of this study are included within the article (and any supplementary files).

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Authors' Contributions

Conceptualization: Z Z and M R; Funding Acquisition; Z Z and M R; Investigation: S C, Z

Z, M R and A S; Methodology: S C, M R and A S; Project Administration: Z Z and M R; Writing—Original Draft Preparation: S C and M R; Writing—Review and Editing: Z Z, M R and A S.

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Conflicts of interest

The authors declare no conflicts of interest.

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General discussion, conclusions and perspectives

3.1 General discussion and conclusions

ES can assist wound healing. However, the research on the fundamental mechanisms of cellular response to ES has fallen behind, which has hindered the full exploitation of the clinical potential of ES. On the other hand, newly developed intrinsically conductive polymers can make ES more efficient. But the poor handling and mechanical properties limited their applications. This thesis firstly aims to prepare a PPy membrane that can be practically used and so fill the gap between a lab material and the material that can be used in medical devices, and secondly aims to understand how ES influences skin keratinocytes in the context of wound healing, which can help to fill the gap between fundamental science and clinical research. So, in this thesis, the author studied the cellular and molecular response of keratinocytes, which is the principal cell type of the epidermis, to the ES that was delivered through a PLLA/PU fiber-reinforced pure PPy membrane. In the material aspect, the author prepared a conductive polymer membrane that can be easily handled, and established an ES protocol specifically optimized for this type of conductive substrate. In the biological aspect, the author cultured the keratinocytes on the conductive membrane and studied the cell viability under ES, including cell growth/death. The author further evaluated the cellular response to ES from the molecular perspective by investigating the key growth factors, proteins, and signaling pathways. The methodologies used in this thesis comprise polymer chemistry, material engineering, and cellular and molecular biology, highlighting the multidisciplinary characteristics of this thesis. The results filled to some degree the as-mentioned gaps. Further studies both on material and cell biology may lead to a new ES-assisted therapy to treat skin wounds.

3.1.1 About research question and objectives

Cells create transmembrane potentials by pumping ions in and pushing ions out through various ion channels and maintaining a negative potential inside the cells in a resting state. This transmembrane potential allows a cell to function as a battery to supply energy to the membrane-embedded "molecular devices" [330]. The asymmetrical distribution of ion channels in apical and basolateral cell membranes in epithelial tissue sets up a transepithelial potential (TEP) [33]. For example, in the skin, negative potentials at the outer corneum with respect to the inner basal epidermis were recorded, which allows the skin to function as a battery that

supplies energy to skin cells. When damage occurs in the skin, a lateral electrical potential gradient is established between the wound area and the adjacent intact tissue, and this wound-induced endogenous electrical current declines with the restoration of the wound tissue [175]. It has been suggested that the skin's ability to heal itself partly depends on this natural wound current [331-333], because it can attract cells to migrate to the wound center. It is estimated that the average healing rate decreases by 25% without this current [33]. In addition, the injury EFs in normal skin of the 65 to 80-year-olds were measured to be 48% lower than that of the 18 to 25-year-olds [334]. The wound of diabetic skin and other recalcitrant wounds also have a diminished EF and the associated wound current, which could be a factor contributing to the prolonged or impaired wound healing in these patents [208]. The underlying exact mechanism of how cells interact with this endogenous EF is very complex and remain largely unclear. Some hypotheses were proposed, explaining that the cellular components can reorganize in response to the electrical force. For example, positively charged proteins or lipids may be attracted to cathode [56, 335].

In this context, it is possible that wound healing can be boosted by enhancing the EF around the wound bed. In fact, externally applied ES has shown positive outcomes in animal models and *in vitro* experiments on human cells/tissues [21, 261, 279, 280, 328]. According to these studies, ES increased epithelialization, fibroblast migration, and angiogenesis around wounds. The therapeutic use of ES in clinical trials in wound management demonstrated a larger reduction in wound size and an increased healing rate when compared with control groups [280, 333]. The clinical effectiveness of ES in accelerating chronic wound healing also has been reported [336]. However, due to the insufficient understanding on the fundamental mechanisms of cellular response to ES, it is difficult to recommend any optimal ES protocol as a standard treatment based on the current knowledge. At present, ES is only recommended as adjunctive therapy to treat chronic pressure ulcers in Australia, New Zealand, and the United States; and ES is not recommended to be used in regular practice in the UK [336]. Hence, to fully explore the clinical potential of ES, further research is required to ascertain the underlying mechanisms that mediate ES and cellular/tissue changes.

To apply ES, electrodes made of metals (silver, copper, nickel, titanium) have often been used in clinic [21, 337]. Conductive polymers (CPs), which were developed in the 1970s and have been investigated as biomaterials only in recent 20 years, are still the materials used in

lab studies. These CPs are very attractive for the ES application because they combine the ability to conduct electricity with the polymeric nature, making them very attractive as the bioactive materials in tissue engineering and regenerative medicine [31, 302].

For a biomaterial, the stiffness and surface topography play important roles in cell adhesion, migration, proliferation, differentiation, and apoptosis [338-341]. Materials with tissue-like stiffness can reduce the mechanical mismatch between scaffold and tissues or cells, increasing interface communication and reducing scar formation [342]. Typically, a smooth surface is not good for cell adhesion. For example, studies have shown that polymer-coated platinum (Pt) had an improved cell adherence than bare Pt. Moreover, compared with metals, a polymer surface can be chemically modified with physiologically active species to enhance the biocompatibility and functionality, which gives polymers more possibilities in regulating cellular behaviors. The stiffness of various tissues of the human body varies a lot, from a few pascals (Pa) for the brain to dozens of kilopascals (kPa) for the skin and mega-pascals (MPa) for the bone. Keratinocytes have a stiffness of 120 - 340 kPa [343], while fibroblasts 1 - 5 kPa. Therefore, the PPy membrane prepared in our lab, with 100 - 200 kPa in stiffness [344], should have better tissue compatibility compared with metals (stiffness in MPa level) for skin-related applications.

CPs have a broad and tunable conductivity, from 10^{-10} S cm^{-1} to 10^{3} S cm^{-1}, ranging from insulators to semiconductors and to conductors, depending upon the polymerization method, type of dopant, doping level, etc. Metals are good conductors, usually in the range of 10^{5} S cm^{-1} [345]. Human skin has a conductivity from 2.6×10^{-3} S cm^{-1} to 1×10^{-7} mS cm^{-1} [346], which overlaps with that of CPs.

To summarize, considering CPs' biocompatibility (support cell adhesion [347], help cell growth [348] and reduce the inflammation [349]) and electrical compatibility (similar to tissues, low electric current under a required electric potential), it is more advantageous to use CPs than to use metals as the conductive biomaterials.

Among CPs, PPy, polyaniline (PANI), and poly(3,4-ethylenedioxythiophene) (PEDOT) are the most promising ones for biomedical applications. The investigations on PEDOT are relatively more recent compared to those on PPy and PANI. The main challenge of using PANI for biomedical applications is its poor cytocompatibility, poor processability, and lack

of flexibility. PPy has excellent *in vitro* and *in vivo* biocompatibility, very good chemical stability, reasonably high conductivity under physiological conditions, and facileness in synthesis and surface modification [305, 348, 350]. Since the early 1990s, PPy has been substantially studied as a cell growth substrate in various *in vitro* cell culture models. There has been a significant effort focusing on the preparation of PPy for practical applications, such as surface grafting PPy on a PLA/PET substrate or embedding PPy nanoparticles in a PLA/CL matrix. The in vivo biocompatibility of these conductive composites also has already been tested [24, 313, 351].

Our research group has been working on PPy for more than 20 years. Recently a free-standing soft PPy membrane was successfully developed in our lab [329, 352, 353], which breaks down the technical barrier of making large-sized flexible CPs membranes. If cells are cultured on a pure CP scaffold, when ES is applied, all the cells will be uniformly affected by the EF, making ES more efficient.

Therefore, it makes a lot of sense to use the pure PPy membrane as an ES-enabled wound dressing. However, this pure PPy membrane breaks easily and must be reinforced beforehand. Secondly, exploring the cellular and molecular behaviors of skin cells under ES still requires major efforts. This thesis contributed to both material development and biological discovery.

3.1.2 About methods and results

This thesis includes two parts. In the material engineering part (Chapter 1), the aim is to reinforce the pure PPy membrane for ES applications. Electrospun polymer fibers were chosen to reinforce it. Material tests include mechanical, handling, chemical, electrical, and cytotoxicity analysis. The PU/PLLA fiber-reinforced PPy membrane can be handled easily and has good cytocompatibility, and at the same time maintains the conductivity of the pure PPy membrane. In the cell biology part (Chapter 2), the aim is to broaden the boundaries of knowledge on keratinocyte-EF interactions. HaCaT cells were cultured on the reinforced PPy membrane. ES intensities of 100 and 200 mV mm^{-1} and during 6 and 24 h were chosen to stimulate the cells. Evaluations include cell viability/growth, growth factor/cytokine release, and protein production. Positive outcomes had been obtained. The effect of ES on cells was found “memorized” by daughter cells. Finally, a signaling pathway that mediated the EF-keratinocyte interaction was identified.

3.1.2.1 PLLA/PU reinforced PPy membrane

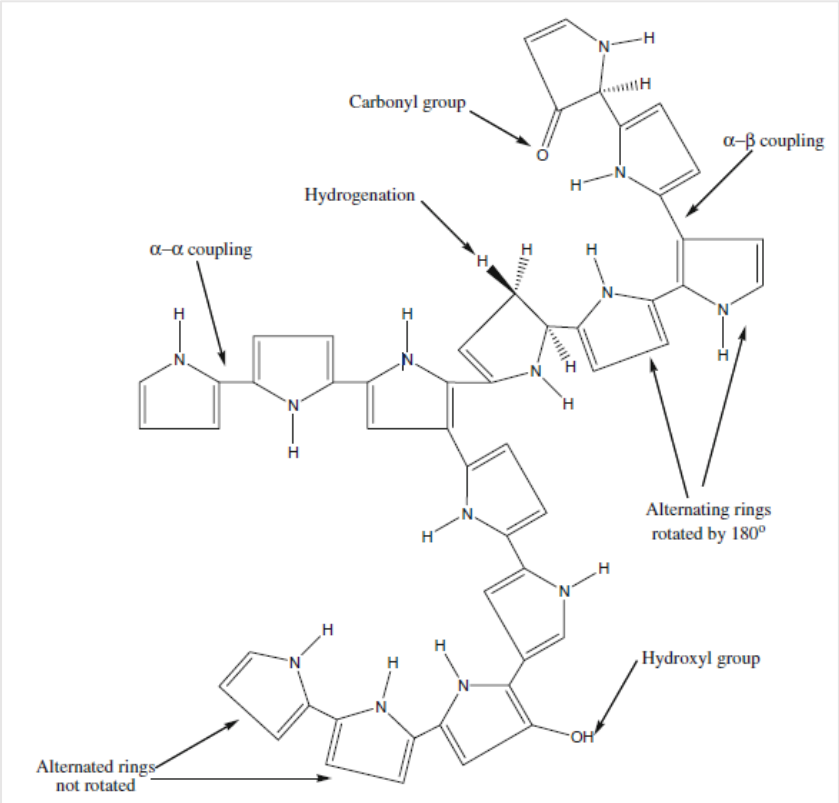

Figure 3.1 Diagram showing various defects in the structure of PPy [354].

It is well known that the application of a polymer is restricted by its physical properties, while the physical properties of a polymer depend on the hierarchical organization of the structures from single molecular to multi-molecular levels. PPy is obtained via condensation reactions among the monomers of the five-membered heterocycle pyrrole with the simultaneous incorporation of counterions [355, 356]. So, an ideal PPy molecule is an array of monomers joined exclusively by α,α' bonds, forming a linear PPy chain with the planar rings at *trans* positions. The ideal charge transport mechanism is through bipolarons, in which two cations transport between every six or eight rings, corresponding to the doping ratios 0.33 and 0.25 respectively [357]. However, the PPy polymers obtained so far in practice are all structurally disordered. Previous studies have proved that PPy contains several types of defects (Figure 3.1) [354, 355, 358], which cause a deviation from chain linearity and planarity, leading to structure disorder. The major source of the defect is the coupling through the β position of pyrrole. The presence of β bonds in addition to the α,α' bonds results in polymer branching and cross-linking. Besides, overoxidation of PPy is an inevitable consequence during PPy chain growth

in a nucleophile medium such as water or by contacting air, because the oxidation potential of PPy is lower than that of pyrrole [359]. In addition, in the traditional chemical oxidation of pyrrole, the polymerization rate slows down as the reactants become depleted in the system, leading to a heterogeneous distribution of the thickness of the PPy [303], which is another defect. So the PPy obtained through chemical polymerization is usually in the form of black powder.

These defects have a significant influence on the properties of PPy. The loss of linearity has important consequences for the structural organization between chains and doping anions. For the PPy with ideal linearity, the hopping of bipolarons along the chain is more effective than hopping along the branched chains. Disorder in PPy leads to other hopping mechanisms at higher organization levels, which is not known at the microscopic level [355, 357]. Crosslinks also make the polymer insoluble. It was demonstrated that 100 - 1000 pyrrole units were the upper limit of the average conjugation length [305]. In fact, PPy is a mixture of molecular chains of very different lengths, particularly in the presence of the β,□ bonds, with the shorter ones being insoluble because of the crosslinks. It should be pointed out that much of our knowledge about the PPy structure is obtained from indirect measurements because it is difficult to find a solvent to yield single crystals, and little information has been obtained from X-ray diffraction studies [357]. Because PPy is sensitive to synthesis conditions, when polymerization parameters such as dopant selection change, the PPy structure also changes.

Given the highly conjugated molecular backbone and the highly disordered molecular structure, the polymer chains of PPy are locked into place through the interlocking network, making plastic flow difficult [340]. Therefore, PPy is mechanically brittle, rigid, and difficult to use alone. Although more homogeneous PPy can be formed through electrochemical oxidation of pyrrole, it is still very hard to eliminate the chemical and structural defects to produce highly ordered PPy. In addition, because PPy is insoluble and thermoset, it is therefore impossible to eliminate the structural defects through after-synthesis modifications. When a force or a displacement is applied to a PPy film, stress concentration occurs easily in the defected areas, and crack propagation will follow until the bulk fracture.

The free-standing and soft PPy membrane synthesized through the template assisted - interfacial polymerization is a breakthrough [329]. This membrane has a homogeneous thickness of around 0.7 μm and can be synthesized in large size, which has not been possible

via any other polymerization methods. However, this membrane still has a poor handling property because of the very low stress and strain at failure.

To improve the tensile strength of the PPy membrane and at the same time not to lose its electrical conductivity, by choosing electrospinning as the fabrication method, a multilayer fibrous reinforcement made of the elastic polyurethane fibres (low Young's modulus but large tensile strain) overlaid with the plastic PLLA fibres (high Young's modulus and tensile strength) was spun to the PPy membrane to prevent the PPy membrane from overstretching.

Electrospinning is an old technique. It, among other fiber fabrication techniques, has become particularly attractive for the manufacture of tissue engineering scaffolds because of the ease to use, the vast possibility in material selection, and the potential to scale up. An electrospinning system simply constitutes a high voltage power supply, a spinneret, and a fiber collector. During an electrospinning process, solvent evaporates, and ultrafine fibers are collected. Almost any soluble polymers with a sufficiently high molecular weight can be electrospun. A variety of polymers have already been successfully electrospun into ultrafine fibers using this technique, including PU and PLLA. Electrospun fibrous mats exhibit good mechanical properties, and have been used as protective textiles, wound dressing materials, drug delivery carriers, etc [360].

Besides the electrospinnability, PU is a synthetic polymer with the urethane groups as the major repeating units. The urethane groups produce strong intermolecular bonds that make PUs useful in applications such as adhesives and coatings. PLLA is a highly crystalline aliphatic polyester and is susceptible to degradation through hydrolysis. Both PU and PLLA have excellent biocompatibility, outstanding mechanical properties and have been used in medical implants [361-363].

PPy membrane is electrically conductive, and that makes it a perfect collector in the electrospun system. The ultimate PU/PLLA reinforced PPy membrane still keeps its biocompatibility and conductivity.

For a cell culture substrate, it is necessary to eliminate all cytotoxic remnants. There are two major sources of the cytotoxic remnants for the PPy-PU/PLLA membrane, *i.e.*, the residual monomers, oligomers, and solvents used in PPy synthesis, and the residual solvents used in

electrospinning. They must be thoroughly removed prior to cell culture since several reports have demonstrated that the poor cell interactions with PPy were due to the missing of the washing step for their polymers before use [356]. The toxic leachable impeded the cell attachment [364]. This work shows that the thoroughly washed PPy-PU/PLLA membranes can be manipulated easily and exhibit good cytocompatibility. The decreased doping ratio and the slightly decreased stiffness are expectable because high doping ratio leads to high conjugation between pyrrole rings (so planar chains) and consequently higher stiffness [312, 357]. Therefore, there was a decrease in backbone stiffness upon de-doping during the washing step. Nevertheless, the long-term electrical conductivity in the culture medium can still meet the requirement of ES. This PU/PLLA reinforced PPy membrane provides us a useful tool for both the fundamental research on cell-EF interactions and the translational research such as ES-assisted wound dressing.

3.1.2.2 ES to keratinocytes

ES is known to affect cells, providing a potential therapeutic approach in the treatment of diseases [178]. There is a growing interest in ES-assisted wound treatment [33], by which cells sense the EF and orient their migration along its direction. Both animal and clinical studies have shown improvement in the healing of skin wounds treated with ES [280, 333, 336]. Due to the complexity of wound healing and the limitations of the ES devices, research on the fundamental mechanisms of cellular response to ES has fallen behind its applications. This work provides an option: a pure conductive polymer membrane-based ES device, through which the cells in contact with the membrane can directly and uniformly interact with the EF, making the study on cell-EF interactions easier.

Keratinocytes are the predominant cells in the epidermis [128]. During wound healing, keratinocytes guide the re-epithelialization. Besides, by secreting important cytokines, chemokines, and growth factors, keratinocytes indirectly participate in other events such as pro-and anti-inflammation, ECM remodeling, etc. Keratinocytes also communicate with other cells such as fibroblasts, macrophages, neutrophils, blood cells, etc., which are essential for wound healing progress [183]. In addition, keratinocytes are sensitive to ES. Previous studies have shown their electrotaxis [18]. However, the current knowledge is far from enough to

understand the interactions between keratinocytes and ES. Therefore, investigating keratinocyte-ES interactions can have significant implications in helping wound healing.

This work chose HaCaT cells, an immortal keratinocyte cell line from adult human skin that has been widely used for skin biology studies [365]. The advantages of HaCaT cells over primary keratinocytes are that using HaCaT cells eliminates the variability between donors and passages. HaCaT cells have a relatively long culture lifetime, making the experimental data reliable and easy to interpret. At the same time, HaCaT cells show similar proliferation and differentiation properties as the primary keratinocytes do. What's more, HaCaT cells can survive without a feeding layer of fibroblasts, providing a scenario to investigate the keratinocytes' response to ES without the inference of other cells. Since endogenous DC EF plays an important role in wound healing, this work still chose a DC mode of ES. The ES intensity and duration were decided by referring to the strength of the endogenous EF and the parameters in previous ES studies [33, 58, 114]. Previous studies about keratinocytes largely focused on cells' instantaneous response to ES, mostly about electrotaxis showing that keratinocytes migrate toward the cathode, and some about cell surface receptors (*i.e.*, EGFR, VEGFR, GPCR, purinergic receptor), ion channels and exchangers (*i.e.*, KCNJ15/Kir4.2, EnaC, NHE1), and the intracellular signaling pathways (*i.e.*, ERK, PI(3)K/PTEN) governing the electrotaxis [63, 73, 277, 366-370].

Keratinocyte migration is vital to close a wound, while its proliferation and differentiation are crucial to re-establish the epidermis [371]. Therefore, this thesis investigated both the instantaneous and the lasting effects of ES on keratinocyte behaviors, focusing on cell proliferation and the secretion of growth factors and cytokines. The outcomes demonstrate that ES can promote keratinocyte proliferation, which can be passed to later generations. The secretion of IL-6, IL-8, IL-1α, GROα, FGF2, and VEGF-A, which are the important mediators for inflammation during wound healing, can be enhanced by ES. It was also demonstrated that keratinocytes responded differentially to the different sets of ES parameters, evidenced by the production of K5/K14 and K10/K13 proteins that are the important markers for keratinocyte proliferation and differentiation, respectively. Finally, our studies revealed that the ERK signaling pathway was involved in the electrical activation of keratinocytes.

From the data of this thesis, a general picture of how keratinocytes interact with ES, from cellular to molecular levels, from immediate to long-term effects, from cell growth to tissue architecture, has been generated. Therefore, this work enriched our knowledge about cellular response to ES's fundamental mechanism and provided the ES protocols that may contribute to tissue re-epithelialization and benefit wound healing.

3.2 Limitations and perspectives

In this study, a novel soft and easy to handle pure PPy membrane was prepared and validated as a cell culture scaffold and as a tool to deliver ES. The PPy-PU/PLLA membrane designed here gives researchers a choice to investigate the mechanisms of how ES activates cells or tissues more efficiently. This membrane may also be used in an ES-assisted wound dressing in clinic. The interactions between keratinocytes and EF reported in this thesis broadened the foundational knowledge and provided a protocol to be optimized before being tested in animal models or in patients. However, there are still a lot of limitations in this study. Firstly, the PPy-PU/PLLA membrane still needs to be handled with care to avoid damage to the membrane. For example, when cells are cultured on the membrane, a scratch assay can't be conducted because it will damage the membrane. Therefore, in the future, studies could be carried out to improve the intrinsic mechanical property of PPy, *i.e.*, to eliminate or reduce the structural defects during PPy synthesis, to control how PPy chains are packed, etc. These studies will be very challenging but highly rewarding as well. Secondly, while ES can be harnessed to manipulate keratinocytes, the knowledge about ES-keratinocyte interactions is still far from fully understood. In clinical practice, the variations between individuals can't be ignored, *i.e.*, the wound-induced EFs are very different in youth and elder, in chronic wound and in trauma. In future, in vitro studies need to be conducted on the cells from different kinds of skin to establish different ES protocols. And different ES modes could be tried to optimize the ES protocol because a long time DC-ES may produce side effects. Thirdly, wound healing is complex in itself. So far, although a great progress has been made, there is still much to be learned. Finally, both in biomedical research and in clinical applications, there is still a lot to be done. A better understanding of the molecular mechanisms will inspire the development of new ES-based treatments.

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Scientific contributions

Publications

1. **Cui S**, Mao J, Rouabhia M, Elkoun S, Zhang Z. A biocompatible polypyrrole membrane for biomedical applications. ***RSC Advances***. 2021;11(28):16996-7006.

2. **Cui S**, Rouabhia M, Semlali A, Zhang Z. Effects of electrical stimulation on human skin keratinocyte growth and the secretion of cytokines and growth factors. ***Biomedical Materials***. 2021;16(6):065021.

Oral presentations and posters

1. **Shujun Cui**, Mahmoud Rouabhia, Ze Zhang. Autoadhesive conductive cryogel as electrode for electrical stimulation to promote skin wound healing. The 37th Annual Meeting of the Canadian Biomaterials Society, Banff, AB, Canada. **May. 25-27, 2022**

(Poster presentation).

2. **Shujun Cui**, Mahmoud Rouabhia, Ze Zhang. Effect of Electrical Stimulation through a Soft Polypyrrole Membrane on the Behaviours of Human Skin Keratinocytes. The 36th Annual Meeting of the Canadian Biomaterials Society, Virtual. **May. 13-15, 2021 (Oral presentation)**.
3. **Shujun Cui**, Mahmoud Rouabhia, Ze Zhang. Study of electrical stimulation through a soft polypyrrole membrane to human skin keratinocytes for wound healing. The 13th Research Day of the Oral Ecology Research Group, Faculté de médecine dentaire, Université Laval, Virtual. **May.6, 2021 (Oral presentation)**.
4. **Shujun Cui**, Mahmoud Rouabhia, Ze Zhang. Polypyrrole membrane reinforced with electrospun Polyurethane and Poly-L-Lactic Acid fibers for biomedical applications. 11th World Biomaterials Congress, Virtual. **Dec.11-15, 2020 (Oral presentation)**.
5. **Shujun Cui**, Mahmoud Rouabhia, Ze Zhang. A soft and electrically stable polypyrrole membrane reinforced with electrospun polyurethane and poly-L-lactic acid fibres for biomedical applications. The 12th Research Day of the Oral Ecology Research Group, Faculté de médecine dentaire, Université Laval, Virtual. **Jul.15, 2020 (Poster presentation)**.
6. **Shujun Cui**, Mahmoud Rouabhia, Saïd.Elkoun, Ze Zhang. A soft and electrically stable polypyrrole membrane reinforced with electrospun polyurethane and poly-L-lactic acid fibres for biomedical applications. The 2019 Annual Meeting of Research Center on High Performance Polymer and Composite Systems, Quebec, QC, Canada. **Dec.13, 2019 (Poster presentation)**.
7. **Shujun Cui**, Mahmoud Rouabhia, Ze Zhang. A soft and electrically stable polypyrrole membrane reinforced with electrospun fibres for biomedical applications. The 35th Annual Meeting of the Canadian Biomaterials Society, Quebec, QC, Canada. **May 21-24, 2019 (Poster presentation)**.
8. **Shujun Cui**, Jifu Mao, Mahmoud Rouabhia, Ze Zhang. Electrospun fibre reinforced conductive polypyrrole membrane for biomedical and energy storage applications. Multidisciplinary Scientific Event in Sustainable Health, Université Laval, **May 23-24,**

2018 (Poster presentation).

9. **Shujun Cui**, Jifu Mao, Mahmoud Rouabhia, Ze Zhang. Electrospun fiber reinforced conductive polypyrrole membrane for biomedical and energy storage applications. The 34th Annual Meeting of the Canadian Biomaterials Society, Victoria, BC, Canada. **May 16-19, 2018 (Oral presentation)**.
10. **Shujun Cui**, Jifu Mao, Mahmoud Rouabhia, Ze Zhang. Electrospun fibre reinforced conductive polypyrrole membrane for biomedical and energy storage applications. The 2017 Annual Meeting of the Research Center on High Performance Polymer and Composite Systems, Montreal, QC, Canada. **Dec.6, 2017 (Poster presentation)**.