Design and Development of Novel Polymeric Microneedle (MN) arrays for MN Characterization, Biomarker Diagnosis and Drug Delivery

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**NANYANG
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UNIVERSITY**

SINGAPORE

**DESIGN AND DEVELOPMENT OF NOVEL
POLYMERIC MICRONEEDLE (MN) ARRAYS FOR
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AND DRUG DELIVERY.**

ZAYIM RAZINA D/O SEENI SYED

SCHOOL OF CHEMICAL AND BIOMEDICAL ENGINEERING

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School of Chemical & Biomedical Engineering

A thesis submitted to the Nanyang Technological University
in partial fulfilment of the requirement for the degree of
Doctor of Philosophy

2019

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research, is free of plagiarised materials, and has not been submitted for a higher degree to any other University or Institution.

10 May 2019

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Date

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Supervisor Declaration Statement

I have reviewed the content and presentation style of this thesis and declare it is free of plagiarism and of sufficient grammatical clarity to be examined. To the best of my knowledge, the research and writing are those of the candidate except as acknowledged in the Author Attribution Statement. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

10 May 2019

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Date

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Xu Chenjie

Authorship Attribution Statement

This thesis contains material from 2 paper(s) published in the following peer-reviewed journal(s) in which I am listed as an author.

Chapter 4 is published as Razina Z. Seeni, Xiaojun Yu, Hao Chang, Peng Chen, Linbo Liu, and Chenjie Xu. Iron Oxide Nanoparticle-Powered Micro-Optical Coherence Tomography for in Situ Imaging the Penetration and Swelling of Polymeric Microneedles in the Skin. ACS Applied Materials & Interfaces, 9 (24), pp 20340–20347 (2017). DOI: 10.1021/acsami.7b00481.

The contributions of the co-authors are as follows:

- Prof Xu Chenjie provided the initial project direction and edited the manuscript drafts.
- I co-designed the study with Prof Xu Chenjie and performed all the microneedle fabrication work at the School of Chemical and Biomedical Engineering. I also analyzed the data.
- All microscopy, including sample preparation and set-up of imaging tools was conducted by Dr Xiaojun Yu in the School of Electrical and Electronic Engineering.
- I prepared the manuscript drafts. The manuscript was revised by Dr Xiaojun Yu and Prof Xu Chenjie.
- Prof Linbo Liu assisted in the collection and provide guidance in the interpretation of the optical coherence tomography data.
- Dr Chang Hao assisted in the schematic drawing of the manuscript.
- Prof Chen Peng provided guidance for understanding and analyzing experimental data.

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The contributions of the co-authors are as follows:

- Prof Xu Chenjie suggested the idea of the project and provided guidance in microneedle fabrication and drug coating technique.
- I performed all the microneedle fabrication and characterization, nanoparticle synthesis, assisted in the imaging process and conducted data evaluation.
- Dr. Mohesh Moothanchery performed the *in vivo* imaging using optical resonance photoacoustic imaging, prepared the animal model prior to imaging and also did analysis of the imaging data.
- I wrote the drafts of the manuscript. The manuscript was revised together with Dr. Mohesh Moothanchery and Prof Manojit Pramanik.

10 May 2019

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LIST OF ABBREVIATIONS

MN	Microneedle
MNs	Microneedles
ISF	Skin interstitial fluid
TDD	Transdermal drug delivery
SC	Stratum corneum
FDA	Food and Drug Administration
PEDOT:PSS	Poly(3,4-ethylenedioxythiophene)-poly(styrenesulfonate)
HA	Hyaluronic acid
ITP	Iontophoresis
PS- <i>b</i> -PAA	Poly(styrene)-block-poly(acrylic acid)
PS	Poly(styrene)
GOX	Glucose oxidase
HRP	Horseradish peroxidase
4-AAP	4-aminoantipyrine
TOPS	N-Ethyl-N-(3-sulfopropyl)-3-methylaniline, and sodium salt
μOCT	Micro optical coherence tomography
PAM	Photoacoustic microscopy
CSLM	Confocal laser scanning microscopy
OR-PAM	Optical resolution-photoacoustic microscopy
PMMA	Poly(methyl methacrylate)
AuNPs	Gold nanoparticles

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ABSTRACT

Advances in microtechnology and biomedical imaging technology has led to extensive research for self-administered and minimally-invasive microneedle (MN) devices to overcome drawbacks of existing transdermal drug delivery modalities. Microneedle mediated drug delivery is an amalgam of the conventional transdermal patch and the hypodermic needle injection. This novel drug delivery method increases the permeability of skin by piercing the stratum corneum barrier to create transient micropores through which drug molecules can passively diffuse. Furthermore, MN treatment is relatively painless and well tolerated by most patients, making it a very realistic technique for clinical implementation.

In the first generation of microneedles (MNs), materials such as silicon, stainless steel, titanium and ceramics have been widely used in MN fabrication. However, several drawbacks such as poor biocompatibility, breakage in skin and need for expensive microelectronics technology for fabrication exists. In the contrary, polymeric MNs have attracted extensive attention due to their excellent biocompatibility and biodegradability. Most importantly, different types of polymers display distinct degradation profiles and swelling properties which can be potentially leveraged upon to fabricate polymeric MNs with different mechanical properties and performance.

Polymeric MNs have shown to be ideal candidates for both transdermal drug delivery (TDD) and skin interstitial fluid (ISF) extraction. Water-soluble and biodegradable polymers have been used in the fabrication of MNs to achieve different drug release profiles such as bolus or sustained drug release. Swellable MNs fabricated using hydrogel-forming polymers have been investigated for its potential in ISF

extraction for clinical biomarker diagnosis. Hence, with the promising outlook of polymer based MN technology this thesis work aims to broaden the scope of existing polymeric MN design and drive the development of novel polymer based MN arrays for clinical applications.

In this thesis, polymers are used in the development of novel MN arrays for transdermal drug delivery and ISF extraction. Specifically, a conductive MN for in situ combination with iontophoresis (ITP) is fabricated for directional and fast transdermal drug delivery in a one-step approach. Most of the current polymeric MN platforms rely on passive diffusion driven concentration gradients to facilitate drug movement from the epidermis into the deeper layers of the skin; delaying the onset of drug effect. The use of conductive and biocompatible polymers; poly(3,4-ethylenedioxythiophene)-poly(styrenesulfonate) (PEDOT:PSS) polymer and hyaluronic acid (HA) in the design of the MN array results in aqueous pathways of low electrical resistance to be created in skin resulting in faster flow rate of drug molecules. This approach offers a new strategy for non-invasive and controlled delivery of drug molecules into deeper tissues underlying the epidermis to result in quicker systemic effect.

Next, a proof-of-concept for a polymer MN-based minimally invasive glucose sensor that can extract ISF and provide colorimetric detection of glucose in a single step is described. Prepared using a poly(styrene)-block-poly(acrylic acid) (PS-*b*-PAA) swellable tip and non-swellable polystyrene core, the MN tips are encapsulated with an assay solution that qualitatively produces a color change from colourless to purple in the presence of glucose. This process is achieved within minutes as it leverages on the polymer properties of the MN tips; it is made up of poly(acrylic acid) polymer which contains ionizable carboxylic acid groups that allow sufficient extraction of ISF. The color change observed on the MN tips is easily correlated to the glucose concentration

and quantified using an ultraviolet–visible spectrophotometer (UV-VIS). Thus, this work strongly illustrates the potential of using the swellable polymeric MN array for quick determination of glycaemic conditions for patients requiring frequent monitoring.

Aside from the development of novel polymeric MN arrays, imaging tools for characterization of MN behaviours such as dissolution and swellability was studied in detail. Current techniques of characterization include mostly *in vitro* and histological studies. The cumbersome and destructive nature of these studies results in poor accuracy and do not permit real time visualization of MN behaviours in skin. For the first time, optical resolution-photoacoustic microscopy (OR-PAM) is shown to be capable of achieving real time and *in vivo* monitoring of the spatial distribution of gold nanoparticles (AuNPs) in mice skin when delivered using poly(methyl methacrylate) (PMMA) MNs. Although optical coherence tomography (OCT) imaging has been widely used in MN imaging, poor backscattering ability of polymers results in low resolution images that require post image processing. Taking this into account, a methodology is developed to use iron oxide (Fe_3O_4) nanoparticles as exogenous contrast agents to enhance image contrast in OCT imaging and shown to enable high resolution and real-time visualization of changes in the profile of poly(styrene)-block-poly(acrylic acid) (PS-*b*-PAA) MNs when inserted into different types of tissues i.e mice skin and human skin. In the future, the imaging techniques can be applied for optimization of MN design parameters and real time visualization of drug release and ISF extraction to optimize device design.

Chapter 1:

Introduction

1.1 Background and Scope

Most microneedle (MN) arrays in pre-clinical/clinical settings are developed as an attractive alternative to invasive and painful hypodermic injections. It has been widely used in vaccine and peptide (i.e. insulin) delivery due to the enhanced skin permeability achieved in a passive manner. However, the clinical realization of MN arrays for small molecule drug delivery suffers from numerous drawbacks. One such important factor lies in the design and fabrication methods of the MN array and its distinct drug delivery properties. The last few decades saw the vast development of MN arrays fabricated using metals, ceramics and silicon. However, the poor biocompatibility of these materials for skin insertion has led researchers to shift to the use of biocompatible and biodegradable natural and synthetic polymers in the design of MN arrays. Aligned in parallel with the trends of the MN field, the work illustrated in this thesis is focused on the design of novel polymeric MNs and development of MN characterization tools in motivation to drive the practical development of polymeric MNs for various transdermal applications.

The development of MN arrays play a major role in the progression of the transdermal route as a viable option for non-invasive systemic drug delivery. However, an apparent bottleneck following MN delivery is the slow passive diffusion of drugs that significantly influences the onset of systemic drug effect. The hypothesis of this project is that a combination strategy involving the use of a novel conductive MN array with iontophoresis is able to greatly reduce the lag time from MN delivery to drug effect. Using a combination of two different polymers that is biocompatible and conductive in nature, the objective is to develop a MN array that can influence the stratum corneum's resistance properties. With a decreased skin resistance, it is postulated that the

application of a driving force (i.e. iontophoresis) should potentially be capable of increased drug transport at a faster rate to deeper tissue layers as a result of increased flow of charges to achieve deeper drug penetration within a short period of time.

In the use of MNs, a novel trend aimed at transdermal biosensing of analytes of clinical interest is rapidly emerging for minimally invasive transdermal interaction with the biological system. Most of the works established in this field demonstrates a two-step approach which utilizes the MNs as skin interstitial fluid (ISF) extraction tools followed by the need for offline analysis to detect the presence of analytes. This limits the translation of such platforms to easy-to-use point-of care devices as trained personnel is required to perform analysis. To envisage the unique possibility of direct collection and detection of analytes, this thesis aims to leverage on the advantages of hydrogel-forming polymers to develop a transdermal MN-based biosensing platform for in situ detection of glucose. It is hypothesized that encapsulation of an enzymatic assay solution in the matrix of the designed polymeric MNs would allow for simultaneous skin interstitial fluid extraction (ISF) and colorimetric detection of glucose present within in a single step approach. If successful clinical translation is achieved, such a development would eliminate the need for expensive biochips based self-monitoring glucose sensors.

During the development of the novel polymer based MN arrays as illustrated above, it was evident that there was a lack of imaging tools for the in situ real time characterization of MN-mediated drug delivery profile and MN swelling process and. Currently, excision of skin samples is performed followed by histological staining which is cumbersome and time-consuming. Imaging technologies can play an important role in the real time monitoring of MN behaviours in *in vivo* conditions. To monitor drug

delivery process in mice tissue, the use of a novel imaging technology optical resolution-photoacoustic microscopy (OR-PAM) is demonstrated for the first time using gold nanoparticles (AuNPs) as a model drug. In the contrary, imaging technologies such as optical coherence tomography (OCT) has been shown to allow real time visualization of MN dissolution in *in vivo*. However, the use of OCT imaging is constrained by poor contrast between MN and tissue surfaces resulting in low resolution images. To observe the real time swelling kinetics of polymeric MNs, it is proposed that incorporating Fe₃O₄ nanoparticles in the MN matrix can result in increased contrast and high-resolution. The improvement in contrast signal in micro optical coherence tomography (μ OCT) imaging of polymeric MNs was investigated and optimized for real time *in-vivo* imaging.

1.2 Objective and Hypothesis

Microneedles have been vastly investigated in the field of transdermal drug delivery and skin interstitial fluid extraction. The key objective of this thesis work is to explore novel polymers and leverage upon its inherent unique physicochemical properties in the design of microneedle arrays for other potential applications.

In the first part of this thesis work, the aim is to develop a novel polymeric MN array to extend the use of microneedles to the field of drug delivery in the oral cavity. The hypothesis of this project is that a combination strategy using conductive microneedles and iontophoresis would result in a synergistic effect to accelerate the passive diffusion of drug molecules from the site of delivery to deeper tissue layers quickly. This increasing rate of diffusion would facilitate for quick drug onset to be achieved necessary in applications such as dental anaesthesia delivery.

In the second part of this thesis work, a novel hydrogel forming polymer is carefully studied in the development of a colorimetric glucose sensor as a point-of-care diagnostic tool. The hypothesis of this project is that the use of an amphiphilic polymer with both hydrophobic and hydrophilic side chains would impart dual functions; firstly the ability to extract skin interstitial fluid and secondly to provide sufficient mechanical strength following fluid extraction. It is necessary the designed MN array retains its structural integrity for simultaneous *in situ* determination of glucose concentrations in the extracted fluid.

Lastly, in the development of novel microneedle arrays, there is a lack in the availability of research tools for non-invasive, real time and *in vivo* characterization of key parameters (i.e. MN penetration depth, drug release profiles etc.) that are necessary in the design optimization. Therefore, in the last part of this thesis work the use of

different imaging modalities for non-invasive and real time MN characterization is studied. Although optical coherence tomography (OCT) imaging has been shown to evaluate parameters such as penetration depth and dissolution rate of polymeric microneedles, it suffers from limited resolution. As such, it is hypothesized that the use of iron-oxide (Fe_3O_4) nanoparticles as an exogenous contrast agents will improve the resolution of optical coherence tomography (OCT) imaging of polymeric microneedles, therefore allowing the real time visualization of interstitial fluid extraction using polymeric microneedles in tissues.

At the same time, the potential of a novel imaging modality optical resolution photoacoustic microscopy (OR-PAM) is also investigated for imaging of polymeric microneedles. The hypothesis of the project is to use OR-PAM to obtain photoacoustic signals from gold nanoparticles that are delivered using polymeric MNs to characterize the real time drug diffusion process and drug penetration depth in skin tissue. The use of such imaging tools to provide high resolution *in vivo* visualization with greater ease and accuracy would benefit researchers developing polymer based microneedles for novel applications.

1.3 Dissertation Overview

This thesis introduces the potential of using different types of polymers in the design and fabrication of MN arrays for drug delivery and transdermal biosensing applications. To drive the development and facilitate the design of novel polymeric microneedles arrays, two novel imaging technologies specifically optical resolution photoacoustic microscopy (OR-PAM) and micro optical coherence tomography (μ OCT) are also discussed.

The following gives detailed description of the thesis work described in each chapter:

Chapter 1 introduces the background and rationale for the research work performed. The objectives and hypothesis of the thesis are stated to set clear directions.

Chapter 2 provides a concise review on skin anatomy, the importance of skin barrier properties in the development of transdermal drug delivery approaches and a literature review on microneedles and iontophoresis techniques.

Chapter 3 establishes the design and development of a novel conductive polymeric MN array that is capable of further accelerating iontophoretic drug delivery for delivery of small molecule drugs into deeper tissue layers.

Chapter 4 introduces the concept of using a swellable polymeric MN array for simultaneous in situ ISF extraction and glucose detection. The chapter also describes the potential of incorporating a colorimetric glucose detection sensor with the designed MN array.

Chapter 5 discusses the technical aspects of applying imaging modalities such as OR-PAM and μ OCT in the characterization of behaviours such as MN swelling and drug release profile.

Chapter 6 concludes the thesis work and entails a detailed discussion on the future works of the different technologies presented herein.

Chapter 2:

Literature Review

2.1 Skin Structure and Need for Transdermal Delivery Approaches

2.1.1 Significance of Skin as a Barrier

For decades, the most common routes of drug delivery were classified as the oral and parenteral routes. With advantages such as pre-determined doses, portability and patient self-administration oral route remains a conventional mode of drug delivery for small molecule drugs [1]. However, for larger therapeutics such as peptides and proteins that are easily degraded by gastrointestinal proteases when delivered orally, the gold standard of drug delivery is via hypodermic needle injections [2]. The invasive nature of injections necessitates administration by trained professionals whilst the pain and phobia associated with needle pricking results in poor patient compliance [3]. The inherent limitations of these common drug delivery routes have led to extensive research for alternative drug delivery modes.

Transdermal drug delivery (TDD) has made an important contribution as an emerging alternative to oral delivery and hypodermic injections. The clinical value of drug delivery through the skin lies in potential attributes such as providing a non-invasive route of administration with an ease of access, bypassing the gastrointestinal (GI) tract and avoidance of first-pass hepatic metabolism [4, 5]. This results in an increased drug bioavailability that can facilitate continuous drug administration enhancing patient compliance. For example, in the treatment of skin disorders, transdermal delivery allows direct access to the diseased site which greatly results in decreased systemic toxicity.

Despite the rapid growth in transdermal drug delivery technologies, as of 2015 only 20 transdermal drug formulations had been approved by the Food and Drug

Administration (FDA) (**Figure 2.1**) [5]. While researchers have recognized the potential of using the intact skin as a port of drug delivery to the human body; the skin poses an incredible barrier to the ingress of materials and only small quantities of a drug is able to penetrate over a prolonged time. It has been shown that delivery of drugs with molecular weight of 500Da and more through skin results in poor skin absorption that prevents therapeutic levels to be achieved [6].

Figure 2-1: Total number of US FDA-approved transdermal drug formulations over time [5].

For transdermal delivery to become amenable for use with a wider range of next generation therapeutic agents, a clear understanding of the mechanisms associated with the barrier properties of the skin must first be developed and overcome. Thus, for achieving successful drug therapy via the skin, it is essential to understand the

parameters that affect the permeability of the skin barrier skin anatomy and its implication on drug-of choice and method of delivery.

2.1.2 Skin Structure and Drug Penetration

As the largest organ in the human body and making up approximately 2m² of surface area and 10% of body mass, the skin plays a vital role in protecting the human body against pathogens by creating a physical barrier from the external environment. The anatomy of skin is made up of three primary layers: the epidermis, the dermis and the subcutaneous tissues (**Figure 2.2a**) [7]. There are also several appendages present within the layers; hair follicles, sweat and apocrine glands and nails. Other key cells present within the epidermis layer include melanocytes, Merkel cells and Langerhans' cells (LCs) which play a vital role in the pigmentation, sensation and immunity functions of the skin respectively [8, 9].

Figure 2-2: Skin structure and biology: (a) The skin is composed of three key layers: epidermis, dermis and subcutaneous layer. (b) The different layers and cell-cell junctions in the epidermis layer [7]. (c) Permeation across the SC is primarily limited by lipids which fills the spaces between corneocytes. Lipids filling the spaces between corneocytes are arranged in a bilayer structure, with hydrophobic tails on the inside and

polar head groups on the outside. Lipids are mainly cholesterol (yellow), free fatty acids (blue), and ceramides (green) [5, 10].

The outermost epidermis layer is subdivided into several layers: stratum corneum (SC), stratum granulosum, stratum spinosum and stratum basale just above the dermis layer (**Figure 2.2b**) [11]. The stratified epidermis layer is made up of 10-20 μ m thick SC mainly composed of densely packed and highly keratinized dead cells. The SC layer represents the primary barrier to drug permeation and can be described using a “brick and mortar” model. It consists of corneocytes (bricks) which are surrounded by an extracellular milieu of lipids (mortar) such as ceramides, free fatty acids and cholesterol organized in lamellar crystalline bilayers (**Figure 2.2c**) [12, 13]. Drug permeation through skin can occur through three major pathways; intercellular (through the lipid bilayers), intracellular (through the corneocytes) and transcellular (through the appendages present) [14, 15]. The lipid-rich nature of the SC prevents the excessive loss of fluid from the body and greatly favours the delivery of only lipophilic and low molecular weight drug molecules. This poses a significant challenge to administering therapeutics via the skin either for local cutaneous effects or as systemic therapy following their entry into superficial dermal capillaries [16]. The remainder of the epidermis layer has a thickness of 50-120 μ m and can be described as an avascular stratified squamous epithelium with cell-cell junctions and associated cytoskeletal proteins that present a secondary barrier to drug permeation. The epidermis is separated by a basement membrane from the dermis; a 1–2mm thick connective tissue which serves as a supporting matrix containing a rich capillary bed [17]. Beneath the dermis, there is the subcutaneous fat layer which is rich in triglycerides and provides support for the epidermis and dermis.

The stringent requirements pertaining to the barrier function primarily by the SC layer of the skin limits the number of drug molecules that can be potential candidates in TDD. The major challenge in TDD lies in increasing the number of drug molecules that can be delivered through skin. Thus, the emergence of novel techniques for skin permeation enhancement is highly necessary for the continued success of this field to widen the transdermal market for new therapeutic indications.

2.2 Transdermal Drug Delivery Approaches

In the past several decades, substances have been placed on the skin for therapeutic effects and, in the modern era, a variety of topical formulations are being developed for treatment of localized medical conditions [18]. TDD has received vast attention as an attractive alternative to conventional oral delivery and hypodermic injection. TDD is defined as a painless method of applying a drug formulation onto intact and healthy skin. It can be intended for either local drug delivery to the skin itself such as in the case of local anaesthetics for skin numbing purposes or used to achieve non-invasive systemic drug distribution targeting the central nervous system (CNS). In such transdermal formulations, the drug initially penetrates through the SC and then reaches the dermal layer and becomes available for systemic absorption via dermal microcirculation [5, 19].

TDD systems are non-invasive, generally inexpensive and can provide drug release for long periods of time (up to one week). As such, it allows the comfort of self-administration and greatly improves patient compliance [4]. The global market for TDD reached \$7 billion in 2015, with an increase of \$238 million just within a year [20]. Over the years, TDD has advanced and evolved through three generations. The first generation consisted mainly of transdermal patches of lipophilic, low molecular weight drugs efficacious at low doses. Transport of the active drug component took place by passive diffusion across the skin - the very basis of TDD with no enhancement. Suitable drug candidates in first-generation TDD approach presented key problems such as low oral bioavailability and desire for less frequent dosing [4, 21]. Examples include the development of TDD patches of nicotine and testosterone which are commercially available [22].

To further improve skin permeability to expand the scope of transdermal drugs, second generation TDD systems focused on the use of skin penetration enhancers as driving forces for transdermal transport of large hydrophilic drugs that cannot undergo passive diffusion. Some of these techniques includes the use of chemical enhancers, non-cavitational ultrasound and iontophoresis. The second generation TDD systems target reversible disruption of the SC barrier or act as an additional driving force in drug penetration [23]. One such example is the development of an iontophoretic system for the delivery of a charged drug molecule lidocaine [24]. Although, significant enhancement was achieved in increasing drug delivery across the SC, a balance had to be strike between drug delivery and protecting deeper tissues from damage. Hence, most second-generation TDD systems have only successfully advanced into clinical practice in the delivery of small molecule drugs for localized delivery, dermatology and cosmetic applications [3, 4, 19].

Drug	Indication	Delivery technology	Clinical phase	Company
AB-1001	Nausea and vomiting	Passive	Phase 3	Abeille Pharmaceuticals (Princeton, NJ, USA)
Acyclovir	Herpes labialis	Iontophoresis	Phase 2	Transport Pharmaceuticals (Framingham, MA, USA)
Buprenorphine	Pain	Passive	Phase 3 ^a	Purdue Pharma (Stamford, CT, USA)
Fertility hormone	Female infertility	Iontophoresis	Phase 1	Vyteris/Ferring (Fair Lawn, NJ, USA/Parsippany, NJ, USA)
Granisetron	Nausea and vomiting	Passive	Pre-registration	Prostrakan (Galashiels, UK)
Heat-labile enterotoxin of <i>E. coli</i>	Travelers' diarrhea	Skin abrasion	Phase 2	Iomai
Human growth hormone	Growth hormone deficiency	Thermal ablation	Phase 1	TransPharma Medical/ Teva Pharmaceuticals (Lod, Israel/ Petach Tikva, Israel)
Influenza vaccine	Influenza prophylaxis	Microneedles	Pre-registration	Becton Dickinson/Sanofi-Pasteur (Franklin Lakes, NJ, USA/Lyon, France)
Insulin	Diabetes mellitus	Thermal ablation	Phase 1	Altea Therapeutics (Atlanta, GA, USA)
Insulin	Diabetes mellitus	Vesicular carrier	Phase 2	Phosphagenics (Melbourne)
Ketoprofen	Osteoarthritis	Heat enhancement	Phase 3	ZARS (Salt Lake City, UT, USA)
Parathyroid hormone (1-34)	Osteoporosis	Microneedles	Phase 2	Zosano (Fremont, CA, USA)
Sufentanil	Chronic pain	Passive	Phase 2	Durect/Endo (Cupertino, CA, USA)
Testosterone	Female sexual dysfunction	Metered dose transdermal spray	Phase 2	Acrux/VIVUS (West Melbourne, Australia/Mountain View, CA, USA)
Testosterone	Male hypogonadism	Chemical enhancer (SEPA)	Phase 2	MacroChem (New York)
Testosterone	Hypoactive sexual desire disorder	Passive	Pre-registration ^a	Procter & Gamble/Watson (Cincinnati/Corona, CA, USA)
Triamcinolone acetonide	Dermatoses	Chemical enhancer (AzoneTS)	Pre-registration	Echo Therapeutics

Table 2-1: Representative transdermal drugs in clinical development using third generation TDD technologies. Adapted from [4]. Copyrights by Nature Publishing Group.

In the third generation, advanced technologies enabled TDD of a variety of drugs including macromolecules (DNA, proteins) and virus-based vaccines through targeted permeabilization of the SC layer [25]. This led to stronger disruption of the SC barrier and enabled more effective transdermal delivery of hydrophilic molecules, therapeutic proteins and vaccines without affecting the deeper tissue layers in skin. Physical disruption of the SC was successfully achieved by using novel chemical enhancers, cavitational ultrasound, thermal ablation, electroporation and microneedles [4, 26]. The growing collection of clinical trials of third-generation TDD systems that have advanced through phase 1 safety trials and into phase 2 and 3 studies of efficacy motivates the development potential of such novel systems for enhanced skin permeability (**Table 2.1**).

Third-generation TDD systems exhibit special promise due to its ability to successfully achieve a suitable balance between effective transdermal delivery and safety to the skin. Techniques such as microneedles, thermal ablation and microdermabrasion increases skin's permeability by forming large micron-scale defects in the SC layer without the need for an added driving force [19, 27]. Although the mechanisms are all different, these methods aim to create 'holes' in the SC layer to allow drug molecules to pass through. The size of disruptions generated by the various methods range are large enough to permit the transport of small molecules to larger macromolecules without causing skin damage of clinical significance. Reports to date have shown that these techniques are well-tolerated by patients and do not result in significant damage to living cells in the viable epidermis and dermis; attributed to skin's self-renewal properties following scarring/infection [4, 19].

Each of the different techniques has its advantages and disadvantages (**Table 2.2**). In the development of TDD systems, it is important to establish a balance between increased transdermal permeability with factors such as safety, patient compliance and cost. Each of these techniques is likely to suit the needs of different applications depending on the desired outcome. For techniques such as electroporation and iontophoresis that do not substantially change the skin barrier but achieve controlled drug dosing, it could be worthy to investigate the potential of these techniques in combination with other methods that increase skin permeability such as the use of microneedles. Thus, the combination of two different enhancers might be the most effective strategy.

Delivery method	Increased transport	Sustained delivery	No pain/irritation	Low cost/complexity
Hypodermic needle	XXX	XX	X	XXX
Chemical enhancers	X	XXX	XX	XXX
Iontophoresis	XX	XXX	XXX	X
Electroporation	XX	XXX	XX	X
Ultrasound	XX	XXX	XXX	X
Microneedles	XX	XXX	XXX	X
Jet injection	XXX	X	X	X
Thermal poration	XX	XXX	XXX	X

Table 2-2: Comparison of efficacy of different TDD approaches. (X-Low efficacy, XX-Moderate efficacy and XXX- High efficacy) Adapted from [19]. Copyrights by Nature Publishing Group.

Overall, TDD offers compelling opportunities to address the low bioavailability of many oral drugs, the pain of injections and the limited controlled-release profile. Building off the successes of third-generation physical enhancers, it is critical and necessary for further scientific and technological advances that enable targeted

disruption of stratum corneum while protecting deeper tissues for an increasingly widespread impact on medicine.

2.2.1 Microneedles

The concept of microneedles (MNs) was proposed as early as in the 1970s, but was only realized in 2000s with the vast development of the microelectronics industry [28]. A microneedles (MN) is a micron-sized needle with a height of 10-2000 μ m and a width of 10-50 μ m [29, 30]. The overarching motivation for using MNs in transdermal drug delivery is that it can provide a minimally invasive means to surpass the SC barrier and enable drug molecules to penetrate through the epidermis layer to the dermal tissue directly and into the microcapillary bed for systemic absorption. In general, MNs can increase skin permeability by creating micron-scale pathways with several key advantages such as no pain, ease of administration, avoidance of first-metabolism and enhanced drug efficacy which can result in dose reduction [31, 32]. MN-mediated transdermal drug transport relies on passive diffusion driven by a difference in concentration gradient [33].

Over the past decade, different types of MNs have been proposed for the delivery of a wide range of therapeutics [34]. The early designs of MNs started from solid MNs that are fabricated mainly using materials such as silicon and metal (**Figure 2.3a**). It is used to painlessly pierce the skin to create holes as a pretreatment followed by the application of an extended-release patch for passive drug delivery. Hollow MNs, on the other hand are comparable to regular hypodermic needles but are shorter in length are also commonly fabricated using materials such as silicon, metal and glass [31]. The mechanism of action resembles that of traditional injections where pressure is used to deliver a liquid formulation of the drug through the predefined hollow bores of the MNs into the skin (**Figure 2.3b**). However, the use of materials such as silicon and glass in the fabrication of MNs suffers from several setbacks such as biocompatibility issues,

breakage inside tissue, the need for intricate multi-step processes and highly specialized and expensive machining tools [35].

Figure 2-3: Types of drug delivery methods to skin using MNs: (A) Solid MNs are used to create residual holes in skin before application of drug. (B) Hollow MNs inject liquid drug formulations into the skin via pressure through its bores. (C) Coated MNs results in drug coating to dissolve upon contact with the aqueous environment. (D) Dissolving MNs encapsulated with drug formulations dissolves to rapidly release drugs upon insertion into skin. (E) Hydrogel-forming MNs integrated with a drug reservoir create transient microchannels in skin for prolonged drug delivery. Adapted from [34]. Copyrights by Journal of Controlled Release.

This has led to a multiplicity of technologies aimed at overcoming this shortcoming. MN fabrication methods have evolved from expensive clean room sculpting of silicon/glass structures to low-cost manufacturing methods such as micro-molding [36]. The use of polymers in the fabrication of MN arrays poses several practical advantages such as low cost of fabrication as it requires the use of simple fabrication techniques such as micro-molding, cheap and widely available raw materials in comparison to expensive silicon wafers and readily scalable to mass production [31, 36-38]. This led to further advancements in MN designs such as coated MNs, dissolving MNs and hydrogel-forming MNs.

Coated MNs are usually fabricated from polymers with strong mechanical strength and contain a drug which envelops its surface (**Figure 2.3c**). It is used to pierce the SC of the tissue, where the drug layer dissolves and the active is released into the skin. On the contrary, dissolving MNs are made of biodegradable polymers and polysaccharides with the drug incorporated into the MN (**Figure 2.3d**). The drug encapsulated within the MN is then released into the skin following the dissolution of the MN inside tissue [33, 39, 40]. Finally, the most recently investigated type is hydrogel-forming MNs which are formulated from non-dissolving crosslinked hydrogels and incorporated with a drug-loaded adhesive patch (**Figure 2.3e**). Following insertion into skin, the hydrogel-forming MNs swell due to the presence of aqueous media in the skin creating hydrogel conduits which then facilitate the flux of drug from the drug-loaded adhesive patch into the skin [41-44]. In comparison to dissolving polymers, hydrogel-forming polymers offer the advantage of delivering doses of drugs that is no longer limited to what can be loaded into the needles themselves. Controlled drug delivery rates can also be achieved by modulating the cross-linking density in the hydrogel-forming polymer network.

The use of hydrogel-forming polymers in the design of MNs has also led to remarkable opportunities in the use of MNs for transdermal biosensing. The characteristic swellable property of these MNs allow the possibility of skin interstitial fluid extraction (ISF) making it a potential tool for biomarker diagnosis. It has been shown to be able to collect dermal ISF by crossing the SC barrier and accessing the fluid in the viable epidermis and dermis in a painless, simple to administer and safe manner [45, 46]. In summary, increasingly new research in the MN field is focused on the development of novel polymeric MN arrays for various transdermal drug delivery and biosensing applications.

2.2.2 Iontophoresis

Iontophoresis (ITP) is a transdermal drug delivery technique that mainly utilizes a low-voltage current as a driving force to transport polar and ionic molecules across the SC. It is commonly stated that ITP mainly provides additional electrochemical driving force for drug transport across skin, rather than increasing the permeability of the skin [21]. The controlled delivery afforded by ITP sets it apart from other technologies. The amount of drug delivered can be controlled by current magnitude, duration of current application and the area of the skin surface in contact with the active electrodes. Other key advantages include an improved onset time and also a more rapid offset time [47]. Currently, ITP is FDA-approved for applications such as delivery of lidocaine for local anaesthesia [48], delivery of pilocarpine to induce sweating in cystic fibrosis diagnostic [49] and in the treatment of hyperhidrosis [50].

Figure 2-4: Schematic diagram showing the flow of drug molecules during iontophoresis. Adapted from [51]. Copyrights by © [2014] IEEE.

An ITP system consists of a power source, a microprocessor and two electrodes (a negatively-charged anode and positively-charged cathode). When the powered electrodes are applied to the target tissue, the generated electric field forces charged molecules to move down their electromotive gradient (**Figure 2.4**). The positive ions

within the tissue are driven away from the cathode and toward the anode. Similarly, the negative ions are repelled from the anode and migrate towards the cathode based on the principle that like charges repel one another [52]. Thus during iontophoresis, if delivery of a positively charged drug is desired, the charged drug is dissolved in the electrolyte surrounding the cathode. On application of an electromotive force, the drug is repelled and moves across the stratum corneum towards the anode, which is placed elsewhere on the body. The movement of drug ions between the electrodes occurs through the skin and communication between the electrodes along the surface of the skin has been shown to be negligible [53].

ITP enhances TDD mainly by the following mechanisms; the ion-electric field interaction that provides an additional force which drives ions through the skin and secondly, electro-osmosis that is generated by the preferential movement of mobile cations and anions present within the SC [54]. Charged drug molecules move across the skin mainly via electrophoresis whereas uncharged molecules can be moved by the electroosmotic flow of water [55]. However, in the delivery of positively charged drug molecules, both electrophoresis and electro-osmosis takes place. This is because the skin is negatively charged at physiological pH and hence, it acts as a cation-selective membrane favouring the movement of cations through anodal iontophoresis [56].

The total iontophoretic flux across skin can be considered to be the sum of the two transport mechanisms – electro-repulsion and electro-osmosis [57]. The contribution of electro-repulsion and electro-osmosis to the total flux of a molecule is dependent on its physicochemical properties. As a general rule, it is suggested that electro-repulsion will decrease with molecular weight and as a consequence, electro-osmotic transport becomes predominant for larger molecules (>1000 Da) [58]. Various factors such as pH, current magnitude, drug concentration and molecular size influence

the total iontophoretic flux. As electro-repulsion is influenced by the charge of the drug, the ideal pH would be one where a large proportion of the molecules is in its ionized form [59]. With a high drug concentration, greater flux will be present as drug diffusion is a function of its concentration [60]. Although an increasing current produces an increase in iontophoretic transport, the effect starts to plateau at higher current levels [47]. It is also highly necessary that a maximum current that achieves a maximum delivery rate does not result in skin irritation and pain [61]. With reports identifying the presence of erythema under the electrode application site the irritation, it correlates that adverse effects are not substantial as long as the current is low.

Because one of the limitations of iontophoresis is that it does not change the skin barrier substantially, it can be combined with third-generation TDD systems such as MNs which are able to disrupt the skin barrier. Considering that such combination strategies are an effective way to overcome the limitations of ITP, the combination of two enhancers has growing potential for enhanced transdermal delivery.

2.2.3 Combination Strategy: Microneedles and Iontophoresis

In recent years, MN technology in combination with other drug delivery enhancing methodologies has been investigated, with the aim of achieving synergistic benefits. It has been suggested that MN application to skin results in the creation of aqueous pathways of low electrical resistance [62]. This observation anticipates the potential of combining MN treatment with ITP to achieve synergistic enhancement in transdermal drug delivery with the added benefit of controlled drug delivery rate. Considerable research works have shown that the combination of these two techniques leads to an increased rate of transdermal delivery for a range of drug molecules.

For example, Lin *et al.* investigated the delivery of antisense oligonucleotide (ODN) by using Macroflux microprojection patch technology. Transdermal delivery of ODN via passive diffusion, Macroflux patch and Integrated Macroflux patch with ITP was studied on hairless guinea-pigs. The sample treated with integrated Macroflux patch and ITP had the highest concentration and therapeutically relevant amount of ODN present in the skin [63, 64]. More recently, Noh *et al.* developed screw-shaped grooves on the surface. Following, pretreatment with MN, anodal iontophoresis was applied for a duration of 6 hours. In comparison to ITP or MN treatments alone, a 2-fold and 6-fold higher drug permeation was observed respectively [65].

To investigate the effect of drug molecular weight (MW) on skin permeation when MN is used in combination with ITP, Wu *et al.* used acupuncture needles of micron-dimensions for pre-treatment of rat skin before iontophoresis application. This resulted in a significantly greater amount of drug accumulation when compared to intact skin after 30 minutes of application duration. The study also concluded that larger MW drugs have a longer lag time and the contribution of ITP to the enhancement of drug

permeation through MN-pre-treated skin increases with increasing MW [66]. Another group, Garland et al. developed dissolvable polymeric MNs and used in combination with ITP to compare skin permeation of low and high MW drugs. The cumulative amount of FTIC-BSA (low MW) delivered across neonatal porcine skin after a 6 h period was found to be approximately 110 μ g for MN alone and 326 μ g for MN in combination with anodal ITP ($p < 0.001$). In comparison, it was observed that the extent of macromolecule delivery was significantly enhance. The cumulative amount of insulin (high MW) in the skin was found to be approximately 227 μ g and 462 μ g for MN and MN in combination with ITP, respectively. This study shows the promising potential of using drug loaded soluble polymeric MN arrays for electrically controlled transdermal delivery in a simple, one-step approach [67]. Chen *et al.* further demonstrated the suitability of dissolving polymeric MN for combination with ITP. The transdermal delivery of insulin administered through unilamellar nanovesicles is delivered using a combination of dissolving MN loaded with the nanovesicles and ITP in diabetic rats. The permeation rates of insulin from nanovesicles with the facilitation of ITP is increased by 3-5 folds in comparison to those without the facilitation of ITP [68].

In summary, it is apparent that most studies has focused on the use of either solid MNs or hollow MNs that are fabricated using metals such as stainless steel for pre-treatment of the SC barrier. Solid MNs are used to puncture holes prior to the application of an electrically conducting drug formulation whereas hollow MNs facilitate drug movement through its central bore. However, upon the removal of the MNs the pores created within the skin can potentially close or in the case of the hollow MNs, the hollow bore can potentially become blocked by compressed dermal tissue during MN insertions. This can often lead to decreased iontophoretic drug flow; thus negating the

length of benefit that may be obtained through the use of ITP. In light of these, the use of drug loaded dissolving polymer MNs and hydrogel forming MNs can be a promising alternative. In addition to overcoming such drawbacks when used in combination with ITP, it also promotes the feasibility of a one-step strategy for enhancing transdermal drug delivery.

Chapter 3:
Design and Fabrication of a Novel
Polymeric Conductive Microneedle Array
for Transdermal Delivery of Drugs
to Deeper Skin Layers

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(Manuscript in preparation)

3.1 Introduction

Microneedles (MNs) have been actively investigated in the last two decades as a physical enhancement technique in transdermal drug delivery platforms. MNs physically disrupt the stratum corneum (SC) barrier to create microconduits that allow drug molecules to pass into the viable epidermis with ease. The micro-dimensions of MNs prevents contact with nerve fibers and blood vessels that reside primarily in the dermal layer. Hence, the principal benefit of using MNs is the promise of highly patient compliant pain-free delivery of both small and large molecular weight active pharmaceutical ingredients.

Depending on the type of MNs used, drugs can be delivered in a variety of ways such as by coating on MN tips, encapsulating in the polymeric matrix of MNs, or injecting through the bores of hollow MNs [69]. Yet, despite the type of drug delivery method, most often the delivered drug molecules are localized around the delivery site within a depth of 50-1000 μm depending on the dimensions of the MNs used. This results in a concentration gradient that then drives drug molecules to move from the site of drug deposition in the epidermis into the deeper layers of the skin [70].

Thus, the process of MN-mediated drug delivery results in slow and non-directional passive diffusion and is only suitable when local accumulation effect of drug in skin is required. On the contrary, in the design of transdermal drug delivery systems targeting systemic circulation drug; this poses challenges such as lack of therapeutically relevant dose concentration and delay in the onset of drug effect. To overcome this limitation, researchers have proposed the combination strategy of MN with iontophoresis (ITP) to accelerate drug diffusion process. ITP involves the use of a small electric current ($\leq 0.5 \text{ mA/cm}^2$) to direct ionic and polar molecules across the highly

lipophilic stratum corneum. This technique allows precise control of the drug delivery rate to be achieved by varying the magnitude of the applied current, duration of current application and the area of skin exposed to current [71]. Both *in vitro* and *in vivo* studies have shown that the combination of these two techniques in one approach can lead to a significantly increased transdermal delivery rate of vaccines, small drug molecules and larger peptides/hormones.

The physical impairment of the skin following MN insertion has shown to synergistically enhance the effects of ITP to resulting in increased skin permeation [72]. In another perspective, it can be understood that the inherent physicochemical properties of skin play a major role in influencing the effect of ITP. Iontophoretic flux is often determined as a sum of both electro-migration and electro-osmosis. By altering skin's resistance properties, the electroosmotic flow can be manipulated to result in an optimization of iontophoretic drug delivery. Skin's top layer epidermis mostly consists of dead and dry cells that results in a characteristic resistance value of 1,000-100,000 Ω/m (dry skin); making it a poor conductor. Thus in this work, the potential use of a novel conductive MN array fabricated using a biocompatible and conductive polymer; hyaluronic acid (HA) and (poly(3,4-ethylenedioxythiophene) polystyrene sulfonate (PEDOT:PSS) to significantly influence skin's resistance and subsequently result in a quicker and accelerated drug diffusion into deeper skin layers when used in combination with ITP is presented (**Figure 3.1**).

Figure 3-1: Conductive HA/PEDOT:PSS MNs for transdermal drug delivery with iontophoresis. (A) The double-layered HA/PEDOT:PSS MN array showing the distribution of Cy5 dye is inserted into the skin (B) In skin, the MN tips rapidly dissolve to distribute the dye molecules in the epidermis layer. (C) Application of electrodes on skin surface to provide a low-voltage current resulting in the migration of the drug into the dermis layer.

PEDOT:PSS is investigated as a potential conductive polymer in the design of dissolvable conductive MN array in this study due to the following reasons. PEDOT:PSS polymer displays unique properties; they merge the positive properties of metals and conventional polymers. It is able to conduct charge, have high electrical and optical properties and most importantly it is soluble and flexible that allows processing with a greater ease [73]. It has been widely investigated in biosensing and bioengineering applications such as nerve grafts, heart muscle patches and neural electrodes [74-77]. Promisingly, PEDOT:PSS polymer has shown to be of good biocompatibility and low cytotoxicity on epithelial cells, neural cells, neuroblastoma cells and fibroblasts [78-82].

The use of PEDOT:PSS polymer as an interface material between the electrodes and skin tissue upon MN dissolution is shown to significantly contribute to a decreased resistance of the skin surface. The presence of PEDOT:PSS in the MN matrix increased the conductivity of HA MNs by 2 folds and reduced the electrical

resistance of mice skin by 30% when deposited in the epidermis layer of skin. Furthermore, the delivery of Cy5 dye into the dermis layer of mice skin when treated with HA/PEDOT:PSS MN and iontophoresis with a current flux density of 3 mA/cm² for a short duration of 3 minutes. To further illustrate this phenomenon of deep drug penetration effect, doxorubicin was loaded into the MN and delivered to melanoma tumor model. The accumulation of the drug in the tumor tissues when conductive MN was used in combination with ITP shows the promising potential of the designed drug delivery platform.

3.2 Materials and Methods

HA-Oligo Degraded by Hyaluronidase (miniHA™) was purchased from Bloomage Freda Biopharm Co. Ltd. (China). Poly(3,4-ethylenedioxythiophene)-poly(styrenesulfonate) (PEDOT:PSS) (1.3wt% dispersion in water) and doxorubicin hydrochloride drug was obtained from Sigma-Aldrich, Singapore. Cyanine5 NHS ester dye was purchased from Precision Technologies Pte Ltd. Melanoma B16-F10 cells were purchased from ATCC (Singapore). Phosphate buffered saline (1X) (PBS) was purchased from Lonza (Singapore). Fischer galvanic iontophoresis unit (Model MD-2) with accessories were purchased from Innomed Pte Ltd. (Singapore). Red dot resting ECG electrodes was purchased from 3M (Singapore). All reagents were of analytical grade and used without further purification.

Fabrication of HA/PEDOT:PSS MNs

The conductive MN array was fabricated via a micromold-based method. Firstly, an inverse-replicate polydimethylsiloxane (PDMS) micromold was made from a pyramidal microneedle stainless steel master structure consisting of 100 needles in a 10x10 array with a height of ~700µm, tip radius of 5µm and a base width of ~300µm (Micropoint Technologies Pte Ltd (Singapore)). This was achieved by pouring PDMS (10:1 w/w ratio of prepolymer to curing agent) over the microneedle master structure before degassing in a vacuum oven followed by curing at 70°C for 2 hours and peeling off from the mold. The obtained PDMS-micromold was then repeatedly used in the fabrication of the MNs. Later, 0.5g of mini-HA was thoroughly mixed with 1ml of aqueous solution containing 5% (w/v) PEDOT: PSS. The resulting solution was poured onto the plasma-treated PDMS mold surface and centrifuged in a swinging bucket rotor (SCANSPEED 1580R, LaboGene) at a speed of 4,000 rpm for 5 minutes to completely

fill the mold cavities. The excess solution remaining in the mold surface was then carefully removed using a glass slide. After overnight drying at room temperature, a second layer of 0.5g/ml of mini-HA solution was added to create the back layer of the MN patch. The MN-array patch was air dried overnight and gently peeled off from the PDMS mold; following which it was stored at 4 °C in an un-humidified condition.

Characterization of HA/PEDOT:PSS MNs

The mechanical strength of the patch was evaluated using Instron 5543 Tensile Meter. Using a 5-mm-diameter flat-head stainless steel cylindrical probe, a vertical oriented force was applied at a constant speed of 0.5 mm min⁻¹ perpendicularly to the MN tips facing upward on a flat-rigid aluminium-plate. The displacement was measured until a pre-set maximum force of 8 N per MN array was achieved. The actual skin insertion ability of the HA/PEDOT:PSS MN patch was examined with fresh porcine cadaver skin. Using 5wt% PEDOT:PSS loaded HA/PEDOT:PSS patch as an example, the MN patch was pressed into the porcine skin with about 1.5 N of force for 1 minute. The skin tissue was fixed in 4% formaldehyde and stained with hematoxylin and eosin (H&E) under standard protocols to examine the presence of micropores in the porcine skin.

Measurement of Skin Resistance

The influence of conductive MNs on mice skin resistance was examined with a handheld multimeter (Escort EDM-169). Firstly, the hair on the dorsal skin of the mice was shaved off and thoroughly cleaned using ethanol. The skin was left to dry before the application of electrodes to measure dry skin resistance value. Next, MNs with different PEDOT:PSS concentrations was applied onto the mice skin for 1 minute. The

residual left on the skin surface was wiped off before the electrodes were applied. The relative distance between the cathode and anode electrodes was maintained at 1 cm apart.

Conductivity of HA/PEDOT:PSS MNs

The I-V characteristics of HA MN and 5wt% HA/PEDOT:PSS MN were measured using a Keithley 2400 SourceMeter. Output current was measured at set voltage from 0 to 1V and at reverse bias with 0.01V intervals. Electrical conductivity was computed using the following formula $\sigma=1/\rho$ based on the slope of the linear regression in the ohmic region (resistance R) and resultant resistivity $\rho=R*(A/d)$, where d is the thickness of the MN array (measured for each MN array with a vernier caliper) and A is the surface area of the electrodes. I-V curves were acquired on $n = 3$ samples per condition [83].

Iontophoretic apparatus set-up

The same iontophoretic apparatus set up was employed for both *in vitro* and *in-vivo* experiments. The commercially acquired 3M electrodes were cut up into 1 cm x 1 cm squares and connected with the device through the crocodile clips.

In vitro Cy5 delivery with HA/PEDOT:PSS MNs

Cy5-loaded MNs were inserted into 1.4wt% agarose gel of 1 cm thickness for 30 seconds for complete dissolution of MN tips followed by the removal of the base layer from the hydrogel surface. After the back layer of the MN patch was removed, electrodes were placed on the opposite sides of the agarose gel with the cathode specifically placed on the MN insertion site. A low voltage current of current flux density of $3\text{mA}/\text{cm}^2$ was applied for 3 minutes. The treated agarose gel was visualized

using Confocal Microscope LSM 800 and quantification of the penetration depth of Cy5 dye was obtained using Image J.

In vivo Cy5 delivery with HA/PEDOT:PSS MNs

All animal studies were performed in compliance with the guidelines set by the Institutional Animal Care and Use Committee (IACUC), NTU (ARF-SBS/NIE-A0326). 6-weeks-old C57/Bl6 male mice (InVivos Pte. Ltd., Singapore) were randomly chosen and anaesthetized using 2% isoflurane in oxygen. The hair in the dorsal region of the mice was shaved and depilated before performing the experiment. A MN patch is inserted into the shaved dorsal region of the mice by pinching the back skin with a thumb and an index finger for 1 minute until the complete dissolution of HA took place. The back layer of the MN patch was then removed and the cathode electrode was placed on the MN-treated area. The anode was placed 1 cm away from the MN treated area. The current (3mA) was applied for 3 minutes and imaged with IVIS. Finally, the mice were euthanized and the skin was excised for further analysis.

In vivo transdermal delivery of DOX in melanoma tumor using HA/PEDOT:PSS MNs

Melanoma disease model was prepared on C57/Bl6 mice through subcutaneous injection of 1×10^6 B16-F10 melanoma cells into its flank. Incubation was given to allow tumor to reach ~5mm in diameter prior to any treatment. MN arrays were prepared containing 5 μ g of DOX and pressed onto the tumor for 2 minutes. Following which, anodal iontophoresis using 3mA was performed for test groups for 1.5 minutes. The mice were then euthanized and skin/tumor tissue at treatment site was excised for imaging and histology. Using the intravital imaging system: IVIS® Spectrum CT (PerkinElmer, Singapore Pte Ltd), fluorescence readings of ROI were taken at respective channels DOX: 515/595nm, cy5.5: 675/695nm with Living Image 4.0

software and normalized against autofluorescence from untreated skin and background surface. Next, the skin was removed and 0.25g of tumor was weighed and homogenized in 2mL PBS at 15krpm for 1 minute with Scilogex D160 homogenizer. The DOX content present in each treatment group is characterized by fitting against a standard curve of known DOX concentration, following normalization against untreated tumor sample. $N \geq 3$ was performed for all groups.

Histology analysis of tissue

The skin tissue/tumor samples were embedded in Optimum Cutting Temperature (O.C.T. Sakura® Finetek) and then sectioned to 10 μ m thickness using Leica CM3050S Cryostat. The mice skin tissue samples were either stained with 1:250 Hoechst 33342 (ThermoFisher) in PBS for fluorescence imaging or with Hematoxylin and Eosin (H&E) under standard protocols for further skin tissue analysis. For melanoma tumor samples, confocal laser scanning microscopy (Confocal microscope LSM 800) was used to image DOX contained in the different skin/tumor samples.

Statistical analysis

Each experiment was repeated at least in triplicates. Statistical tests (t-tests or one-way analysis of variance (ANOVA)) were carried out to obtain P-value significance. Microsoft Excel was used to perform the t-tests. One-way analysis of variance (ANOVA) with post-hoc analysis was performed using a web-based statistics calculator from http://astatsa.com/OneWay_Anova_with_TukeyHSD/.

3.3 Results and Discussion

3.3.1 Fabrication and Characterization of HA/PEDOT:PSS Conductive MNs

The MN array is fabricated using a two-step soft lithography technique incorporating a conductive polymer, poly(3,4-ethylenedioxythiophene)-poly(styrenesulfonate) (PEDOT:PSS) with low molecular weight hyaluronic acid (HA) for the MN tips and only HA polymer was used for the MN base. The therapeutic agent to be delivered is encapsulated in the HA/PEDOT:PSS MN tips during the first step. Different MN arrays were prepared from mixtures of HA and PEDOT:PSS in which the weight percentage of PEDOT:PSS was tuned between 0-15% (**Figure 3.2a**). Increasing concentration of PEDOT:PSS resulted in a greater fraction of the MN tips to be completely filled with the polymer represented by the prominent increase in the black color of the MN tips.

The obtained MN patches displayed MNs with a pyramidal structure of height $\sim 500\mu\text{m}$, a base width of about $\sim 300\mu\text{m}$ and interneedle spacing of about $450\mu\text{m}$ in a 10×10 array (**Figure 3.2b**). To show the feasibility of drug encapsulation in the HA/PEDOT:PSS MNs, Cy5 dye was used as a model charged drug due to its distinct blue color and fluorophore properties. Using confocal laser scanning microscopy, the cross-section profile of the MN tips was obtained. The presence of Cy5 dye was found throughout the MN tips with no Cy5 present in the base (**Figure 3.2c**). It was observed that towards the sharp ends of the MN tips, little or no fluorescence was observed. It could be determined that the black color of PEDOT:PSS polymer led to the quenching effect of the Cy5 dye. To determine the quenching effect of PEDOT:PSS particles on the fluorescence of Cy5 dye, different MNs were prepared with same amount of Cy5 dye but different weight percentage of PEDOT:PSS. In comparison to HA MN, it was

observed that an increasing concentration of PEDOT:PSS led to a greater decrease in the fluorescence intensity (**Figure 3.2d**). Therefore, it is important to note the drug encapsulation efficiency is not compromised with increasing PEDOT:PSS concentrations.

Figure 3-2: (A) Optical microscope images of MN arrays: (i) HA MNs. (ii) HA MNs loaded with Cy5 dye. (iii) 5wt% HA/PEDOT:PSS MNs loaded with Cy5 dye. (iv) 10wt% HA/PEDOT:PSS MNs loaded with Cy5. (v) 15wt% HA/PEDOT:PSS MNs loaded with Cy5. Scalebar: 500µm. (B) Optical image of HA/PEDOT:PSS patch. Scalebar: 0.5cm (C) Confocal scanning laser microscopy image showing the distribution of Cy5 dye in 5 wt% HA/PEDOT:PSS MN. Scalebar: 100µm. (D) Quantification of Cy5 dye in different MNs to determine quenching effect of PEDOT:PSS particles. (E) H&E stain of MN-treated porcine skin. Scalebar: 500µm. (F) Mechanical test characterization of HA/PEDOT:PSS MN arrays. Load versus displacement curves and

(G) Young' modulus measurements of HA/PEDOT:PSS MN arrays of different PEDOT:PSS concentrations.

Next, to determine if HA/PEDOT:PSS MNs are strong enough to achieve skin penetration without breaking, the actual skin insertion capability was assessed using fresh porcine cadaver skin. Taking 5wt% HA/PEDOT:PSS patch as an example, a thumb press was sufficient for the MN to penetrate into the skin tissue. The subsequent histology study reveals the successful penetration of the MN through the epidermis layer. It is notable that the MN does not reach the dermis layer and only penetrates up to a depth of approximately 150-200 μ m as the MN tips quickly dissolve upon contact with skin ISF present in the epidermis layer (**Figure 3.2e**).

The mechanical strength of the conductive HA/PEDOT:PSS patch was further analysed with an axial compression test. MN patches with various PEDOT:PSS concentrations (i.e. 0, 5, 10 and 15 wt%) showed slight variations in its respective load versus displacement profiles which indicates that the mechanical strength is influenced by PEDOT:PSS loading (**Figure 3.2f**). With an increasing PEDOT:PSS concentration, greater compression of the MN tips was observed with increasing force applied. However, all the MN arrays tested were able to withstand an axial load of 8N without fracture indicating enough strength for skin penetration. Furthermore, the young's modulus of the HA/PEDOT:PSS MN patches was also evaluated in comparison to HA MN. Increasing concentrations of PEDOT:PSS resulted in decreasing Young's modulus (**Figure 3.2g**). This indicates that HA/PEDOT:PSS MN becomes less stiffer and more softer when a greater fraction of the MN tips is encapsulated with PEDOT:PSS. In the design of the HA/PEDOT:PSS MNs, the necessary mechanical strength and stiffness for skin penetration is provided by the HA polymer.

To understand the electrochemical properties imparted by the use of a conductive polymer in the design of HA/PEDOT:PSS MN array, the conductive properties of HA MN following the incorporation of PEDOT:PSS was studied. Firstly, blank HA/PEDOT:PSS MN was inserted into mice skin and ITP was performed. The skin sample was then sectioned to observe the distribution of PEDOT:PSS inside the tissue upon MN dissolution. It is visualized that most of the PEDOT:PSS polymer (blue-black color) resides at the site of MN dissolution on the epidermis layer. The application of current did not result in any significant movement of PEDOT:PSS particles into the deeper dermis layer (**Figure 3.3a**).

Figure 3-3: Influence of PEDOT:PSS on HA MN array's conductive properties: (A) Distribution of PEDOT:PSS polymer following dissolution of MN tip (the deep blue color) in the epidermis layer of mice skin. Scalebar: 100μm. (B) Measurement of skin resistance using a multimeter before and after insertion of HA/PEDOT:PSS MNs of different PEDOT:PSS concentrations. (C) I-V curves of HA MN and 5wt% HA/PEDOT:PSS MN.

To study the effect of the presence of PEDOT:PSS material in the skin interface on skin resistance, a multimeter was used to measure the change in the skin's resistance before and after MN application. Taking the HA MN as a comparison to the HA/PEDOT:PSS MN, it was clearly observed that the skin's resistance greatly reduced

(Figure 3.3b). However, an increasing concentration of PEDOT:PSS did not result in any further significant decrease in skin's resistance. The electrical conductivity of these dry HA/PEDOT:PSS MN electrodes was also measured by studying the I-V characteristics of the HA/PEDOT:PSS MN patch. The output current was measured at set voltages from 0 to 1V and at reverse bias with 0.01 V intervals **(Figure 3.3c).** The electrical conductivity was calculated based on the slope of the linear regression in the ohmic region. The use of PEDOT:PSS in the design of the MN array led to a greater than 2.5-fold increase in the conductivity of the HAMN array. Therefore, 5wt% HA/PEDOT:PSS MN was chosen for subsequent studies due to its mechanical strength and high conductivity. In addition, as the MN dissolves upon contact with skin it is important that minimal amount of residue is left inside the skin as this would hinder future clinical translation.

3.3.2 In vitro transdermal delivery of Cy5 using HA/PEDOT:PSS MNs

The potential of conductive HA/PEDOT:PSS MNs for accelerated transdermal drug delivery in combination with ITP was firstly investigated using 1.4wt% agarose gel as an *in vitro* replica model of skin with similar water content and integrity. The MN patches loaded with Cy5 dye were inserted into agarose gel covered with parafilm for 30 seconds. Cy5 dye was chosen as a model drug to represent a small molecule drug with a positive charge. Upon complete MN dissolution, the base patch and parafilm was removed before application of cathodal iontophoresis.

Figure 3-4: Distribution of Cy5 dye in 1.4wt% agarose gel: (A) 2D planar view of Cy5 penetration in 1.4wt% agarose gel when treated with HA MN, HA MN + Iontophoresis and 5wt% HA/PEDOT:PSS MN + Iontophoresis. (B) Representative 2D view of Cy5 penetration in the z-axis in agarose treated with HA MN + Iontophoresis and 5wt% HA/PEDOT:PSS MN + Iontophoresis respectively. (C) Quantification (n=10) of average depth of Cy5 penetration in agarose of different treatment conditions. (**p < 0.01)

The results obtained indicated the synergistic effect of the combination treatment with conductive MN and ITP on drug penetration depth (**Figure 3.4a**). When only HA MN was inserted into agarose gel, it was observed that the MNs penetrated to a depth of $\sim 200\mu\text{m}$ and resulted in a bolus delivery of Cy5 dye. Most of the dye was present throughout the depth of MN penetration indicating no/slow diffusion. When ITP was applied in combination with HA MN, it could be observed that the drug molecules were driven further into the agarose gel up to a depth of $600\mu\text{m}$. This observation concludes the presence of an additional driving force as a result of ITP application rising from electro-repulsion effect. When the HA MN was replaced with a conductive HA/PEDOT:PSS MN, a stronger iontophoretic flux was present as observed by the bulk movement of most of the dye particles from the surface of dissolution. An average penetration depth of $800\mu\text{m}$ was observed suggesting a 1.5-fold increase in the drug penetration depth (**Figure 3.4b**). The short duration of 3 minutes application time with a current density of $3\text{mA}/\text{cm}^2$ was sufficient to allow stronger charge flow that resulted in a significantly increased penetration depth. A further analysis of Cy5 distribution along the depth of the agarose was performed using images obtained in the z-axis plane (**Figure 3.4c**). It can be visualized that combination of conductive MN and ITP achieved twice the penetration depth.

3.3.3 *In vivo* transdermal delivery of Cy5 using HA/PEDOT:PSS MNs

Following the observation of increased depth of penetration of Cy5 in agarose gel; to validate the suitability of the MN platform in *in vivo* a mice model was chosen to study the possibility of accelerated Cy5 penetration using HA/PEDOT:PSS MN and ITP. The MN patch was thumb pressed into the mice skin for 1 minute to allow enough time for complete dissolution of MN tips into the skin and subsequently the back layer was removed. Following which the cathode electrode was placed in situ of the MN application site while the anode electrode was placed at 1cm apart for 3 minutes at a current flux density of $3\text{mA}/\text{cm}^2$ (**Figure 3.5a**). The presence of a fluorescence signal in the mice skin tissue imaged using IVIS at a wavelength of 650/670nm suggests the presence of penetrated Cy5 dye. The presence of PEDOT:PSS polymer in the tissue did not result in a positive signal at the measured wavelength and thus the signal observed in treated samples is quantified and interpreted as a true signal of Cy5 dye. From the representative IVIS images, mice samples treated with conductive MN and ITP displayed stronger Cy5 fluorescence intensity. Mice skin samples treated with 5wt% HA/PEDOT:PSS MN resulted in approximately 11-fold greater Cy5 signal than compared to the skin treated with HA MN only (**Figure 3.5b & 3.5c**).

Figure 3-5: *In vivo* studies of conductive MN and iontophoresis on transdermal drug penetration: (A) Experimental set-up of MN treatment followed by iontophoresis. (B) Representative IVIS images for all three sets of experiments of mice treated with different MN arrays with iontophoresis. (C) Quantification of IVIS fluorescence signal of Cy5 in treated mice skin tissues normalized by background fluorescence. (D) Representative fluorescent images of mice skin sections stained with Hoechst (blue) and fluorescent Cy5 dye (purple). Scale bar: 100 μ m. (E) Image J quantification of Cy5 fluorescence particle count in epidermis (stratum corneum/epidermis) and dermis layer. Increasing trends of Cy5 count was observed in dermis when the mice was treated with both conductive MN and iontophoresis. (N=5, **p<0.01)

To measure the depth of penetration of Cy5 dye in treated mice tissue, skin sections were obtained and samples treated with 5wt% HA/PEDOT:PSS MN and HA MN was compared. Most of the Cy5 dye (purple signal) delivered was localized throughout the epidermis layer when only HA MN was used with ITP. In contrast, Cy5 dye was distributed throughout the epidermis and dermis layer when 5wt%

HA/PEDOT:PSS MN was used with iontophoresis (**Figure 3.5d**). The deeper penetration of Cy5 dye in skin tissue is in parallel with the observations made in 1.4wt% agarose gel. Further analysis of the Cy5 count in the various regions of the skin (epidermis and dermis) in each treatment groups showed a greater than two-fold higher Cy5 count in skin sections treated with 5wt% HA/PEDOT:PSS MN (**Figure 3.5e**). Although the amount of Cy5 dye encapsulated in all the MNs is the same, the difference in the Cy5 count can be accounted for. Without strong iontophoretic flux, bulk of the dye particles remain in the surface of the treated tissue samples. When the tissues are excised and treated for staining, it is expected that the dye can easily washed away. This coincides with the observation that most Cy5 dye is present beyond the micropores created by MN penetration.

3.3.4 *In vivo* transdermal delivery of DOX in melanoma tumor using HA/PEDOT:PSS MNs

While mice skin is of negligible thickness, it is necessary to evaluate the efficiency of the developed platform to enhance drug delivery to deeper tissue layers. As such, to further illustrate the accelerated drug permeation effect, a melanoma tumor model was used. A tumor of approximately 5mm was created on the dorsal region of mice and treated with different MN patches loaded with an anti-cancer therapeutic doxorubicin (DOX). The MN patches were thumb pressed onto the tumor for 2 minutes to allow complete dissolution of MN tips. As DOX is a negatively-charged drug, anodal iontophoresis was performed (**Figure 3.6a**). Following treatment, the tumor was excised with the skin tissue attached and IVIS was performed to observe DOX delivery. For tumor samples treated with 5wt% HA/PEDOT:PSS and ITP, a strong DOX signal was clearly observed as shown in the representative IVIS images (**Figure 3.6b**). The blank sample (treated with HA MN without DOX) exhibited autofluorescence signal and hence was used in the normalization of DOX fluorescence signal intensity for treated samples. Similarly, quantification of DOX signal from tumor sample treated with 5wt% HA/PEDOT:PSS showed greater than two-fold higher fluorescence intensity indicating that greater amount of DOX was able to permeate the tumor tissue (**Figure 3.6c**).

Figure 3-6: *In vivo* studies of DOX-loaded conductive MN array and iontophoresis on transdermal drug penetration in melanoma skin model: (A) Experimental set-up of MN + iontophoresis treatment on melanoma tumor induced mice. (B) Representative IVIS images for all three sets of experiments of melanoma tumor treated with different MN arrays. (C) Quantification of fluorescence intensity of treated melanoma tissues. The fluorescence intensities derived from DOX was normalized by background fluorescence. (D) Amount of DOX present in different treatment groups. (E) Image J quantification of DOX particle count in epidermis (stratum corneum/epidermis) and dermis layer in treated melanoma tumors. Increasing amounts of DOX count was observed in dermis when the mice was treated with both conductive MN and iontophoresis. (** $p < 0.01$, * $p < 0.05$)

Next, to analyse the permeation of DOX specifically into the tumor tissue, the surrounding skin was isolated from the tumor samples. The tumor samples from each treated group was then weighed to obtain equal masses and homogenized to calculate the amount of DOX present in each test groups. The results indicate higher amount of DOX present in tumor samples treated with 5wt% HA/PEDOT:PSS MN and

iontophoresis in comparison to samples treated with HA MN and iontophoresis. This clearly points out the synergistic effect of PEDOT:PSS and iontophoresis. This is further implied when samples treated with 5wt% HA/PEDOT:PSS MN with iontophoresis resulted in 1.5-fold greater DOX accumulation in the tumor in comparison to samples treated with 5wt% HA PEDOT:PSS MN without iontophoresis (**Figure 3.6d**).

In addition, to investigate the distribution and penetration depth of DOX in the tumor samples in the different layers (skin and tumor), sections of the treated samples were observed for DOX fluorescence using confocal microscope. It was observed that treatment with both MN and iontophoresis showed presence of DOX in both skin and tumor in comparison to samples treated with MN alone; DOX was observed to be present only in the skin's epidermis layer. Furthermore, for samples treated with 5wt% HA/PEDOT:PSS MN, greater depth of penetration was achieved throughout the entire tumor depth (**Figure 3.6e**). The depth of penetration for the various treatment groups was quantified by measuring DOX particle count at different depths throughout the tumor samples. Only for tumor samples treated with 5wt% HA/PEDOT:PSS MN, the fluorescence intensity beyond the skin periphery ($>200\mu\text{m}$ as indicated by the black dotted line) was observed to be significant and observable up to a depth of $650\mu\text{m}$ into the tumor (**Figure 3.6f**).

The combination of conductive MN and ITP resulted in a stronger iontophoretic flux to be present that resulted in enhanced permeation rates of Cy5 dye and DOX in the respective studies. The use of conductive MNs not only causes a reduced skin resistance, it also provides microchannels that effectively reduce the skin barrier that further contributes to the increase of diffusion coefficient of the small molecules delivered. Both the *in vitro* and *in vivo* studies agreed that the penetration of the Cy5

dye and DOX driven by both conductive MN and ITP resulted in a synergetic effect for transdermal delivery.

3.4 Conclusion

In summary, we have developed a conductive MN array using a conductive polymer (PEDOT:PSS) and a biodegradable polymer (HA). The conductive nature of PEDOT:PSS polymer enables a significant reduction in skin tissue resistance (stratum corneum and epidermis layer) resulting in a stronger flow of charges through the highly innate resistant skin. Leveraging on the increased electromotive force present due to conductive MN application, we further accelerate iontophoretic transdermal drug delivery to achieve deeper permeation of drug molecules for quicker systemic circulation. Given the recent trend of minimally invasive microdevices for transdermal drug delivery, this newly designed platform can potentially open up the possibilities of achieving quicker drug delivery with enhanced efficacy and reduced dosage.

Chapter 4:
Development of a Rapidly Swellable
Polymeric Microneedle Array Integrated
with Colorimetric Glucose Detection

(Manuscript in preparation)

4.1 Introduction

Diabetes mellitus is the most common metabolic disorder across the world. It is related with the abnormal metabolism of blood sugar and defective insulin production [84]. Poor glucose control often results in a myriad of health conditions such as blindness, kidney failure, neuropathy, heart diseases and increased susceptibility to infections that can lead to amputation of lower limbs [85]. In the optimal management of diabetes, frequent and accurate monitoring of blood glucose is a key cornerstone. The gold standard of glucose monitoring lies in the use of handheld glucometers that require repetitive and painful finger-pricks to draw blood samples [86]. This often results in tissue damage and patient discomfort compromising frequent monitoring with reports suggest almost 50% of diabetic patients do not adhere to routine blood glucose measurements [87].

In the development of routine self-monitoring glucometers, blood sampling poses challenges such as inability of patients to draw venous blood themselves resulting in apprehension and poor sample quality of fingerstick capillary blood. Skin interstitial fluid (ISF) is emerging as an attractive alternative to blood sampling due to its vast source of systemic and dermatological biomarkers. In addition, skin is an easily accessible organ that can be easily targeted for ISF extraction. Current methods of ISF collection from skin includes the use of suction blisters, microdialysis and open-flow microperfusion [88-90]. However, these techniques are time-consuming, pose risks to patients, require medical expertise and specialized equipment limiting their clinical use. Hence, simpler and minimally-invasive ISF sampling methods will bring about greater potential for clinical utility of ISF in diagnosis. Thus, extensive efforts have been aimed

at developing non-invasive glucose sensors that rely on optical, electrical, heat, or electrochemical techniques[91, 92].

Continuous glucose monitoring system (CGMS) are commercially available devices for detection of glucose in ISF using amperometric electrochemical technology [93]. It was FDA-approved in 1999 and is used widely as an important tool for detecting episodes of high blood glucose and low blood glucose. The technology consists of a needle-type electrochemical glucose sensor and is inserted beneath skin to access ISF. Studies have shown that increased frequency of glucose monitoring using CGM devices for a week or more contributes to improved glycaemic control [94, 95]. Despite the advantages, the invasive nature of these devices results in patient discomfort limiting continuous use. Furthermore, the placement of subcutaneous sensors is susceptible to surface deposition of proteins and cells (biofouling) which affects sensor accuracy. This requires ongoing calibration with blood glucose values presenting challenges in its clinical application [96].

To overcome such challenges, MNs a miniaturized replica of hypodermic needles in the length of hundreds of micrometers have attracted great attention for transdermal monitoring of biomarkers present in ISF [97, 98]. MN mediated sampling of interstitial fluid (ISF) is emerging as an attractive alternative to blood sampling for a range of analytes, with glucose being a prime target. Due to their short length, MNs provide a minimally invasive method of fluid extraction allowing them to successfully penetrate the SC and gain access to the ISF present within the viable epidermis and upper layers of dermis. This reduces pain, possibilities of infections, sample contaminations and analysis artifacts that may rise in self-administered blood sampling.

Additionally, it has also been shown that glucose concentrations in dermal ISF and finger capillary blood has a high correlation with an estimated lag time between 0 and 45 minutes, and a mean lag time of 6-7 minutes [99]. To account for this, MN- based sensors that are developed for glucose measurements in ISF require calibration to capillary blood glucose. Thus, the integration of MNs for ISF extraction in biosensors presents a novel class of minimally invasive and painless devices for both *in vitro* and *in vivo* measurements of analytes of clinical interest [100].

Different types of MN-based glucose sensors have been developed thus far. Most commonly, hollow and solid MNs are fabricated using materials such as metal, silicon and glass for ISF extraction [101-104]. Hollow MNs extract ISF via capillary action through the microchannels present in the needle orifice. Solid MNs are used to create micropores in skin's stratum corneum and ISF is extracted using external devices such as vacuum. In a recent study by Samant *et al.*, different MN arrays containing submillimetre solid, porous, hollow needles were evaluated for ISF extraction capabilities by penetration into the superficial skin layers of porcine cadaver skin to access ISF via the micropores formed. The results demonstrated successful collection of >1 μ L of ISF within 20 minutes from porcine skin and living human subjects for all types of MN [105].

The use of the MNs for ISF extraction has been greatly focused on the generation of pores to facilitate in ISF sampling. However, the use of materials such as glass and silicon in the fabrication of MNs poses risks such as possibility of breakage in skin and environmental hazards due to poor disposal after usage. In addition, the need for multiple steps (i.e. using vacuum to provide negative pressure) to accelerate ISF extraction further prevents the adaptation on the discussed platforms in clinics. An

emerging alternative is the development of polymeric hydrogel MNs which is capable of ISF extraction in a single step by utilizing its swelling ability in skin. Additionally, the biocompatibility and biodegradable nature of hydrogels results in minimal tissue damage and low environmental hazard risk. Compared with hollow MNs, hydrogel MNs are cost-effective and easy to synthesize.

Caffarel-Salvador et al. developed MNs using poly(methylvinylether-co-maleic anhydride) (PMVE/MA) crosslinked with polyethylene glycol (PEG) and demonstrated successful ISF extraction (0.84mg in 1 hour) for detection of various analytes such as glucose, theophylline and caffeine [106]. More recently, our group proposed the development of swellable MNs fabricated using crosslinked methacrylated hyaluronic acid (MeHA) for quicker and greater volume of ISF extraction (1.4mg in 1 minute). Similarly, the ISF collected using MeHA MNs was used for glucose and cholesterol detection. The reduced sampling time is advantageous in the development of routine point-of-care testing (POCT) as it would greatly reduce anxiety increasing patient compliance [107].

In both the techniques described above, the extracted ISF metabolites are efficiently recovered from the MN patch by centrifugation method for the subsequent offline analysis using commercially available test kits [108]. Despite the successful pioneering works suggesting the potential of hydrogel forming MNs for ISF extraction in a short time without the need for extra devices; offline analysis of metabolites using lab-scale instrument hinders the clinical translation to achieve a real-time POCT. In this study, a hydrogel MN-based glucose sensor is developed that realizes ISF extraction and *in situ* colorimetric detection of glucose in a single step approach is proposed. The use of a block polymer consisting of poly (styrene-*b*-acrylic acid) (PS-*b*-PAA) and polystyrene (PS) in the fabrication of MNs is shown to be capable of fluid extraction.

The MN tips are then incorporated with an enzyme based colorimetric glucose sensor which enables simultaneous ISF extraction and glucose quantification. The incorporation of a colorimetric glucose assay results in a quick color change when glucose is present in the ISF extracted. The observed color intensity on the MN tips is correlated with glucose concentrations and can be interpreted with a pre-determined color scale.

This paves way for a quick test of glucose conditions and can be used by patients at any time of the day. The minimally-invasive nature of MNs also greatly enhances patient compliance and thereby accommodating to the frequent monitoring of their glucose conditions. With closer monitoring of glucose condition on a daily basis, healthcare professionals are able to come up with a personalized treatment for each patient improving quality of life.

4.2 Materials and Methods

Poly(styrene)-block-poly(acrylic acid) (PS-*b*-PAA) with number average molecular weights (M_n) of 26,000 g/mol for poly(styrene) (PS) and 128,000 g/mol for poly(acrylic acid) (PAA) was purchased from Polymer Source Inc (USA). PS pellets with average molecular weights (M_n) of 180, 000 g/mol, D-glucose (G8270), glucose oxidase from *Aspergillus niger* (G6125), peroxidase from horseradish (P8125) and 4-aminoantipyrine (A4382) were purchased from Sigma (Singapore). SeaKem® LE Agarose of gelling temperature (1.5%) 36°C $\pm$ 1.5°C was purchased from Lonza (Singapore). Phosphate buffered saline (1X) (PBS) was purchased from Lonza (Singapore). N-Ethyl-N-(3-sulfopropyl)-3-methylaniline, sodium salt (TOPS) was obtained from Dojindo Molecular Technologies, Inc. (USA). All reagents were of analytical grade and used without further purification.

Fabrication of PS-*b*-PAA/PS MN array

The PS-*b*-PAA /PS MNs were fabricated using PS-*b*-PAA via a solvent casting method as previously reported with slight modifications [109]. Briefly, a female polydimethylsiloxane (PDMS, Dow Corning 184 Sylgard) micromold was made by pouring PDMS mixture (10:1 w/w ratio of pre-polymer to curing agent) into the MN stainless-steel master structure (10x10 array containing MNs of 800 μ m height, 250 μ m base diameter, 450 μ m interneedle spacing and 5 μ m tip radius) (Micropoint Technologies Pte Ltd, Singapore). After degassing in a vacuum oven for 30 min and curing at 70 °C for 2 hours, the PDMS mold was peeled it off. The MNs were made in two steps. Firstly, the swellable MN tips were prepared by filling the PDMS mold with 100 μ L PS-*b*-PAA solution (10% in DMF). Centrifugation (4000rpm, 5min) in a swinging bucket rotor (SCANSPEED 1580R, LaboGene) was used to sink the polymer

solution into the tip cavities. The filled PDMS mold was left in the fume hood for 48 hours at room temperature to allow the evaporation of DMF and formation of the PS-*b*-PAA tip. Next, the backing layer of the MN was formed by melting PS pellets (Mw: 180k) on the top of the PDMS mold at 180 °C under vacuum for 4 hours. Finally, the PS-*b*-PAA /PS MN patch was cooled to room temperature before gently peeled it off from the mold.

Modification of PS-*b*-PAA /PS MNs for the in situ glucose detection

The prepared PS-*b*-PAA /PS MNs were then modified by loading glucose detection assay into the MN tips. Specifically, the glucose detection assay contained four different components comprising of the following optimized concentrations: 16 units/ml glucose oxidase (GOX), 27units/ml peroxidase from horseradish peroxidase (HRP), 2.5mM 4-aminoantipyrine (4-AAP) and 2.5mM N-Ethyl-N-(3-sulfopropyl)-3-methylaniline, and sodium salt (TOPS). PBS (0.01M, pH 7.0) was used as the solvent to prepare the stock assay solution. The ratio of the different components was pre-determined using calibration curves to find a suitable composition with high sensitivity and specificity. The stock solution of the assay solution was stored in the fridge at 4°C refraining from light. The PS-*b*-PAA MN tips were immersed in the assay solution containing the above components for 1 hour and then left to dry overnight in a vacuum filled desiccator. The modified MNs were stored in 4°C in air-tight well-plates covered in aluminium foil.

Mechanical test and skin penetration efficiency test

The mechanical property of PS-*b*-PAA /PS MNs was examined using an Instron 5543 Tensile Meter. A vertically oriented force was applied perpendicularly to the MN tips facing upward on a flat-rigid aluminium-plate, using a 5-mm-diameter flat-head

stainless steel cylindrical probe (at a constant speed of 0.5mm/min). The displacement was measured until a preset maximum force of 8N was reached. The actual skin penetration ability of the MN patch was tested by using a thumb press force of about 1.5N into fresh porcine cadaver skin for 5 minutes and removed. After removal, the morphology changes of MN patch in this process as well as the micro-sized holes on skin were examined by digital microscope (Leica DVM6). The treated skin was stained with Trypan blue to evaluate the uniformity of skin piercing.

Swellability of PS-*b*-PAA /PS MNs in the agarose hydrogel

The swelling ratio of the PS-*b*-PAA /PS MNs was determined by comparing the weight of each MN patch in the dry state (m_o) and after the fluid extraction (m_t). Specifically, the MN patches were pressed onto 1.4wt% agarose gel covered with a layer of parafilm. The MN patches were removed at specific time points (i.e. 1min, 3min and 5min) and weighed (m_t). The swelling ratio was determined using the following equation: % Swelling = $\left(\frac{m_t - m_o}{m_o}\right) \times 100\%$.

Visualization of PS-*b*-PAA tip swelling in hydrogel with micro optical coherence tomography (μ OCT)

A lab-built μ OCT system was employed for imaging. The details of the μ OCT setup are described in a previous publication [110]. On the scanning head of μ OCT, there was a portable window holder with a glass window placed below the objective lens. The glass window's back surface was fixed around 200 μ m above the focal plane. When the MN patch was in direct contact with the agarose hydrogel, the sample light would be approximately focused at the MNs. During the imaging, the agarose hydrogel or tissue sample was placed on the glass window while MNs were placed on a motion

stage such that they could be moved to penetrate into the hydrogel gradually. It is worth noting that another identical glass window was inserted into the reference arm optics for dispersion compensation in the imaging process. When the μ OCT was utilized for MN geometry characterization, both glass windows and the portable window holder were removed from the system.

Setup of correlation curve between color change and glucose concentration for PS-*b*-PAA /PS MN glucose sensors

PS-*b*-PAA /PS MN glucose sensors were tested with a range of glucose concentrations from 1mM to 12mM to identify the correlation between the color intensity of MNs and glucose concentration. In the experiments, 1.4wt% agarose hydrogels containing different concentrations of glucose were prepared. PS-*b*-PAA /PS MN glucose sensors were then gently inserted into the agarose covered with a parafilm for 3 minutes. The color change of MN patch during this process was quantified with a UV-VIS spectrophotometer (550nm).

Biocompatibility of PS-*b*-PAA /PS MN glucose sensor

The biocompatibility of PS-*b*-PAA /PS MN glucose sensor was studied with the MTS Cell Proliferation Colorimetric Assay Kit (BioVision). Briefly, mouse fibroblasts (NIH/3T3 cell, ATCC) grown in the complete cell media (Dulbecco's modified Eagle's medium containing 10% fetal bovine serum), were seeded in a 24-well plate. The cells were then treated with PS-*b*-PAA /PS MNs loaded with different components of glucose assay. The cells were then washed once with PBS and incubated with complete media containing 20 μ L of MTS solution for 2–3 hr at 37 °C. The absorbance was measured at 490nm using an absorbance plate reader (H4 Synergy Biotek).

Statistical analysis

All data were obtained from at least three independent experiments with at least three parallel samples per condition in each experiment and are expressed as means $\pm$ standard deviations (SD). Significance was assessed with ANOVA and Student's t-test. A probability value of $p < 0.05$ was considered significant.

4.3 Results and Discussion

4.3.1 Characterization of PS-*b*-PAA/PS MN

The design of the MN platform involves the use of an amphiphilic block copolymer polystyrene-block-poly (acrylic acid) (PS-*b*-PAA) to achieve a double layered MN design with selective localization of swellable material in the tip region (**Figure 4.1Ai**). The base of the MN array is made up of polystyrene (PS) polymer which imparts the mechanical strength and structural integrity necessary for MN penetration and stability during insertion and swelling of MN tips. This hybrid polymer is chosen to give the MNs the ability to rapidly absorb water as poly(acrylic acid) (PAA) is a well-known super absorbent material that contains carboxylic acid groups which ionizes quickly in the presence of water. The height of the designed MNs was measured to be approximately 700 μ m and the base was 200 μ m and thus capable of penetrating the stratum corneum to reach the ISF present in the viable epidermis and dermis layer without encountering underlying nerves.

Figure 4-1: Characterization of PS-*b*-PAA/PS MN integrated with enzyme based colorimetric glucose sensor. (A) Images of MN patches before and after insertion into porcine skin. (B) H&E stain of PS-*b*-PAA/PS MN treated porcine skin. (C) Comparison

of Load versus displacement curves for PS-*b*-PAA/PS MN arrays before and after incorporation of enzyme based colorimetric glucose sensor. (D) The swelling ratio of PS-*b*-PAA /PS MN patches in the agarose-parafilm skin model at different time intervals. (E) OCT visualization of the real-time swelling kinetics of PS-*b*-PAA/PS MNs in agarose hydrogel.

The designed PS-*b*-PAA/PS MN array was characterized for its ability to penetrate and extract ISF. The actual skin insertion capability of the MN patch was examined by using a thumb press (about 1.5N) to penetrate fresh porcine cadaver skin. Following removal of the MN patch, it was observed that the MN tips adopted a swollen morphology with the structural integrity of the MNs still well-maintained (**Figure 4.1Aii**). The treated porcine skin displayed a complete array of spots created by the insertion of the MN tips (**Figure 4.1B**). To study the influence of glucose assay incorporation into the PS-*b*-PAA MN tips, the mechanical strength of the MNs was evaluated using a compression test. Both the MN patches tested displayed similar load versus displacement profiles indicating that the incorporation of glucose assay solution in MN tips does not influence its mechanical properties (**Figure 4.1C**).

Next, the swelling abilities of PS-*b*-PAA/PS MN patches was investigated by studying the mass change of the MN patch before and after their insertion into 1.4wt% agarose gel. The percentage swellability ratio of the MN patches reached a plateau within 5 minutes indicating maximum swellability potential is reached (**Figure 4.1D**). To visualize the real-time swelling kinetics of PS-*b*-PAA MN, the MNs were inserted into a transparent agarose hydrogel and imaged using μ OCT. It was observed that the tips of the MNs swelled upon contact with fluid in agarose gel whilst the MN tips in air maintained its sharp tip morphology (**Figure 4.1E**).

In comparison to other hydrogel-forming polymers that are capable of fluid absorption such as PMVE/PEG MN and MeHA MN described previously, PS-*b*-PAA/PS MN array was chosen as a suitable candidate for the incorporation of a colorimetric glucose sensor. PMVE/PEG MN has been shown to take a very long time to extract ISF. In the contrary, MeHA MN is able to extract ISF in a few minutes resulting in both the MN tips and MN base to swell up; with the MN tips losing their structural integrity. For this technology, a slow swelling rate and complete swellability of the MN patch is not desirable. Upon fluid extraction, it is necessary that the MN patch should still maintain its structural integrity in order for subsequent colorimetric detection to be achieved. In the design of PS-*b*-PAA/PS MNs, only the PS-*b*-PAA MN tips are capable of swelling upon fluid extraction. The MN base composed of PS polymer is unable to swell and expand in the presence of ISF exhibiting mechanical strength and structural integrity. This ensures that the PS-*b*-PAA/PS MN array can be removed from skin after application for color change observation.

4.3.2 Optimization of Assay solution for in situ glucose detection

Having demonstrated the rapid extraction of fluid using PS-*b*-PAA/PS MNs, the next step was to develop a suitable chromogenic method for detection of glucose. Commonly used components of commercially available colorimetric assays was chosen to prepare a suitable assay. In the presence of glucose molecules, GOX reacts with oxygen to produce hydrogen peroxide. The produced hydrogen peroxide then facilitates the oxidation of HRP which results in the formation of a colored product in the presence of TOPS and 4-AAP (Figure 4.2A) [111]. The use of such an assay solution resulted in the quick formation of a color change from colourless to purple. A fast chemical reaction is highly beneficial because of the short time-scales of analysis required for a practical POCT diagnostics.

Figure 4-2: (A) Schematic of colorimetric detection assay used for glucose sensing. (B) Sensitivity of developed glucose assay solution. (C) Mouse fibroblasts (NIH/3T3) cells treated with different components of the assay solution and the assay solution. MTS cell viability assay performed after incubation with enzymes and dyes for 24 hours.

In the next step, the composition of the different components in the assay solution was determined for measuring glucose concentrations in the 0-10mM range, consistent with concentrations found in the ISF as previously reported [112, 113]. The absorbance value at different concentrations was measured to construct a correlation graph. The chosen concentrations of the different components in the assay solution was optimized to result in a high sensitivity for glucose detection up to 20mM. The high linearity ($r=0.99$) observed in the correlation graph depicts the sensitivity of the prepared glucose assay and its suitability for ISF glucose conditions (**Figure 4.2B**).

The different components of the assay solution are not FDA-approved drugs, and therefore it is important these components do not elicit an immune response when loaded into MNs for transdermal sensing of glucose. Mouse fibroblasts (NIH/3T3) was chosen as a suitable cell line to test the toxic effect of these dyes and enzymes. Cells' viability was examined with MTS cell proliferation assay (OD:490nm) after 24 hour incubation with GOX, HRP, 4-AAP and TOPS solution. The selected concentrations of the different components of the glucose assay did not significantly affect cell viability (**Figure 4.2C**). Therefore, the optimized assay was loaded into the PS-*b*-PAA/PS MNs for subsequent glucose detection experiments.

4.3.3 *In vitro* glucose detection and quantification using PS-*b*-PAA/PS MNs

To test the efficacy of the PS-*b*-PAA/PS MNs to extract ISF and potentially be used as a sensor for detection of clinically relevant biomarkers, an experiment was designed to extract glucose from glucose solution and agarose hydrogel. Following the encapsulation of the assay solution in the MNs, 10uL of glucose solution with varying concentration was added to the patches. Visible color change was immediately noticed for higher glucose concentrations (i.e. 9.7mM) whereas for lower concentrations it took approximately 3 minutes for a complete color formation on the MN patch (**Figure 4.3A**).

Figure 4-3: (A) PS-*b*-PAA/PS MN patches tested in different concentrations of glucose solutions. (B) PS-*b*-PAA/PS MN patches tested in different concentrations of glucose loaded agarose gel. (C) Quantification of colorimetric detection signal of treated PS-*b*-PAA/PS MNs using UV-VIS at an absorbance peak of 550nm.

The experiment was similarly repeated with varying glucose concentrations in agarose hydrogel. The MNs were pressed onto the agarose hydrogel with a gentle pressure; and likewise, the higher concentration of glucose resulted in a quicker change in color (**Figure 4.3B**). However, it is noteworthy that the time taken to observe a color change was much quicker with adding glucose solution directly onto the MN patch then

compared to insertion into agarose hydrogel. Such a phenomenon is observed in the detection of glucose in agarose hydrogel mainly because MNs do not quickly come into direct contact with glucose as it has to achieve penetration of the parafilm layer first which could result in the lag time observed.

The intensity of the purple color change observed for different glucose concentrations is relatively the same regardless of the substrate tested on. This data is crucial to indicate the specificity of the designed MN platform in its potential to accurately extract ISF and detect the relevant concentration present. To further validate the results observed, we used UV-VIS to obtain a calibration curve for the detection of different glucose concentrations. An increasing glucose concentration resulted in a higher absorbance value indicated by the increasing peak (**Figure 4.3C**). The in vitro results presented above illustrates the potential of the device to provide a rapid and facile method to detect glucose after 3 minutes of application time and enables the discrimination between normal glycaemia (4-7mM) and hyperglycaemia (>7mM). Such an integrated device design obviates the need for extraction and further processing of ISF extracted using MNs to obtain a glucose level.

4.4 Conclusion

Blood glucose monitoring in diabetes management can enhance treatment efficiency, alleviate the symptoms, and diminish the complications of the condition. The traditional care for people with diabetes often requires monitoring of blood glucose and insulin injections to maintain normoglycemia. However, such self-administration is often associated with pain and inadequate glucose control. This study serves as a proof-of-concept reporting the development of a rapidly swellable MN array integrated with an enzyme based colorimetric glucose sensor. The aim of MNs is to reduce the invasive nature of existing technologies without compromising on sensor accuracy. The integration of microneedles in biosensors envisages a novel class of minimally invasive and painless biosensors for both *in vitro* and *in vivo* risk-free measurements of analytes of clinical interest.

Using this device, both ISF extraction and real time analysis of glucose concentration can be performed in a single step approach. Owing to the presence of carboxylic acid groups in PS-*b*-PAA polymer, the MN patch can easily be used for both incorporation of enzymes for colorimetric detection of glucose and simultaneous extraction of ISF. The PS core of the MN design provides sufficient strength for skin penetration and subsequent removal after ISF extraction.

In the monitoring of glucose condition, colorimetric detection is compelling and combines well with the portable microneedle platform. It allows for direct outcomes which could be observed by the naked eye without the need for additional sensor technologies. Thus, from a patient perspective, a simple MN-based sensor enacts the possibility of a painless, self-administered POCT device with high accuracy and real-time detection easing self-monitoring of glucose levels.

Chapter 5:

Characterization of Polymeric Microneedle Arrays' Performance using Novel Imaging Modalities

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5.1 Introduction

Microneedles (MNs) have attracted keen interest among researchers because of their applicability in both transdermal drug delivery and ISF extraction. This technology is used to create micropores in the SC barrier to enable the transdermal delivery of a broad range of therapeutics such as large, hydrophilic molecules that cannot permeate intact skin [42, 70, 114]. While MN arrays have been studied extensively for drug delivery into skin, there has been an increasing focus on the use of MNs to access ISF. With reports dictating that ISF contains biomarkers of interest for systemic and dermatological analysis, MNs are being explored as potential tools for extraction of ISF in the development of diagnostic sensors [103, 106, 108, 115]. With the promising outlook of MNs in its various applications, it is greatly anticipated that MN arrays can be utilized for a variety of therapeutic and diagnostic systems.

Recent advances in MN research has led to the development of polymeric MN arrays in contrary to metal, ceramics and silicon counterparts in favour of its nontoxicity, biodegradability and biocompatibility properties. The use of polymers also allows the feasibility of engineering MN arrays with different drug release profiles which is not easily achieved with other types of MNs [45]. In the design and development of polymeric MNs, it is highly necessary for researchers to perform characterization studies to evaluate key parameters such as MN penetration depth, dissolution or swelling behaviours, transdermal diffusion rates and drug distribution profile in skin. Furthermore, design optimization of MN arrays for applications such as extraction of ISF requires real-time monitoring of *in vitro* and *in vivo* swelling kinetics.

To date, research investigating the micropores produced by MNs has primarily been performed *ex vivo* due to their small size and the difficulties associated with *in vivo*

analysis. Commonly-used procedures involve puncturing the skin using microneedles, then using standard histological techniques and optical microscopy to image an excised section [116]. However, these traditional methods are cumbersome and time-consuming requiring the excision and processing of tissue specimens. Furthermore, as skin tissue is elastic it usually does not retain the microstructures generated during MN intrusion. This hampers quantitative data collection such as penetration depth and closure kinetics of microneedle-induced skin pores. Knowledge of these parameters is vital for assessing important transdermal delivery and fluid extraction parameters such as dosage profiles, drug diffusion rates, swellability and skin healing times.

Alternatively, the use of imaging technologies can provide for a non-invasive, *in situ* and real time visualization of MN behaviours in tissue samples making them highly desirable. In this chapter, two different imaging methodologies namely, photoacoustic microscopy (PAM) and micro optical coherence tomography (μ OCT) are introduced as characterization tools for polymer-based MN arrays. These imaging technologies function as a form of non-destructive "optical biopsy" to allow *in vivo* imaging of tissue structure in real time and *in situ* without the need for tissue pre-treatment or removal.

In this study, PAM is shown to be useful in visualizing real time spatial distribution of drug molecules delivered from coated polymeric MNs while μ OCT is used to observe the real time fluid extraction capability of a hydrogel-forming polymeric MN. PAM is described for the first time to be capable of visualizing drug release from MN array. The use of laser light allows for a high penetration beyond optical mean free path (~ 1 mm in skin) with high resolution and has been successful in *in vivo* structural, functional, molecular and cell imaging. Using gold nanoparticles (AuNPs) as a model

drug, clearly visualization of drug diffusion process and spatial distribution is observed in *in vivo*.

In comparison with PAM, μ OCT is limited by its shallow imaging depth and poor contrast between the MNs and the skin tissue. Herein, a methodology is described to use Fe_3O_4 nanoparticles as suitable μ OCT contrast agents to improve the contrast in the imaging of polymeric MNs when inserted into tissue. Whilst, this methodology has proven to be highly effective in using μ OCT for studying of MN-based dissolution and swelling kinetics, it is not well-suited for drug release studies.

The use of these imaging technologies can be extended to the novel polymeric MN arrays described in this thesis. Using PAM, the quick release from the dissolvable conductive MN array can be observed to study the duration necessary to achieve drug delivery to deeper tissue layers. Similarly, with the improved resolution in μ OCT imaging, a non-invasive method can be used to determine the amount of fluid that can be extracted using hydrogel-forming MN arrays. Promisingly, the use of these imaging technologies allows for more accurate determination of key parameters in the characterization of MN behaviours; supplementing the growing interest in polymer based MN arrays.

5.1.1 Photoacoustic microscopy (PAM)

PAM is a fast-growing hybrid *in vivo* imaging modality combining optical contrast with ultrasound resolution [117-123]. It provides penetration beyond optical mean free path (~1 mm in skin) with high resolution (a lateral resolution within tens of microns and an axial resolution within hundreds of microns) and has been successfully applied to *in vivo* structural, functional, molecular, and cell imaging [122, 124-127]. In the imaging process, PAM utilizes a short laser pulse to irradiate the tissues. There will be a temperature rise due to absorption of light by the tissue chromophores (such as melanin, red blood cells, water etc.) which in turn produces pressure (acoustic waves) waves. A wideband ultrasonic transducer receives the acoustic signal outside the tissue boundary for reconstructing the optical absorption map inside the tissue.

PAM overcomes the limitations of other existing optical modalities, combining optical contrast with ultrasound resolution. In PAM, the resolution is not limited by optical diffusion due to multiple photon scattering whereas contrast is related to the optical properties of the tissue. Unlike other imaging technologies such as optical coherence tomography, diffuse optical tomography and fluorescence tomography, PAM does not rely on ballistic or backscattered light. Any light, including both singly and multiply scattered photons, contributes to the imaging signal. As a result, the imaging depth in PAM is relatively large with sustained high spatial resolution. Compared with ultrasonic imaging, PAM has rich intrinsic and extrinsic optical contrasts and is free of speckle artifacts [128].

PAM can be classified into acoustic resolution photoacoustic microscopy (AR-PAM) and optical resolution photoacoustic microscopy (OR-PAM). In AR-PAM, deep tissue imaging can be achieved with weak optical and tight acoustic focusing. Resolving

single capillaries acoustically will need ultrasonic transducers greater than 400 MHz central frequency; but at this frequency the penetration depth will be less than 100 μm . Therefore, tight optical focusing has been used in OR-PAM to achieve higher spatial resolution. OR-PAM can resolve single capillaries or even a single cell [129], and a lateral resolution up to 0.5 μm has been achieved [123, 130, 131]. Although the penetration depth of OR-PAM is limited due to light focusing, it can still image up to a maximum of 1.2 mm inside the biological tissue [123].

In the *in vivo* visualization of MN-mediated drug delivery in skin tissues, imaging modalities such as confocal scanning laser microscopy (CSLM) has been used. For example, in a recent work by Bal *et al.*, CSLM was used to image the diffusion of a fluorescent dye (used as a model drug) through the skin following MN insertion. However, the technique was limited by the penetration depth of approximately 200 μm and the field view of the system [132]. Therefore, we hypothesize that OR-PAM which has superior penetration depth and resolution can be used to observe the penetration of MNs through skin and simultaneously observe the real time delivery of drugs. As a proof-of concept, the skin penetration of PMMA MNs is studied using OR-PAM. Then using gold nanoparticles (AuNPs) as a suitable model drug, the drug diffusion profile and distribution of AuNPs in mice skin following MN-assisted delivery is visualized and quantified. .

5.1.2 Micro-optical Coherence Tomography (μ OCT)

OCT is a well-established and powerful non-invasive three-dimensional imaging technique that uses reflections of light waves to generate cross-sectional images with axial resolutions of 1-15 μm . It typically employs near-infrared light which allows the imaging of tissue structures up to a depth of several millimetres[116]. Currently, OCT is used as a routine imaging tool in the field of ophthalmology [133, 134] and applied in clinical research areas such as skin, dental, endoscopy, and colonoscopy [135-138]. Recently, the development of micro-optical coherence tomography (μ OCT), a new form of OCT; further pushes the imaging resolution down to 1–2 μm , allowing the visualization of a single cell[139].

In light of these, there has been growing interest in applying optical coherence tomography (OCT) to image MNs behaviours for transdermal drug delivery applications [140]. Through utilizing a classical OCT system, Enfield *et al.* reported for the first time the real-time OCT imaging of MN's skin penetration *in vivo* [141]. Similarly, with lab-built OCT systems, Liu *et al.* assessed the dissolving process of silk MNs in different carriers for the first time [142], while Tsai *et al.* investigated the temporal effects of both morphological and vascular changes in mouse skin induced by MNs' insertion [143]. However, it is noteworthy that the images obtained in all these studies displayed only limited contrast, and therefore, the real-time visualization of MNs' penetration and dissolution lacked clarity and resolution. This is mostly due to the weak light backscattering ability of polymers that resulted in poorly-defined structures and image artefacts in the above studies. As a result, post-processing techniques such as addition of false colors was necessary to significantly improve contrast between MN and tissues.

Herein, we describe the suitability of using Fe_3O_4 nanoparticles as OCT contrast agents for use in OCT imaging of polymeric MN based applications. Previously, we report the use of iron oxide (Fe_3O_4) nanoparticles to significantly enhance OCT signal in the imaging of cellular-uptake of Au- Fe_3O_4 nanoparticles. [144] Thus, we hypothesize that the incorporation of Fe_3O_4 nanoparticles in polymeric MNs might help improve the contrast in OCT imaging of MNs' performance. In addition, Fe_3O_4 nanoparticles are biodegradable and widely used in preclinical/clinical studies as magnetic resonance imaging agents and magnetic hyperthermia agents[145, 146]. Thus, the use of magnetic nanoparticles in MNs would bring more potentials in multimodal imaging and therapy without the concern for safety.

In this part of the study, a lab-built μOCT system is adopted to demonstrate the contrast enhancement when Fe_3O_4 nanoparticles are encapsulated in polymeric MNs. With the enhanced contrast, clear distinction between MN and tissue is observed. Such a setup also paves way for real time *in situ* monitoring and quantitative characterization of the swelling properties of polymeric MNs in different biological samples (hydrogel, mouse skin, and human skin).

5.2 Materials and Methods

5.2.1 Fabrication of Polymeric Microneedle Arrays

To exhibit the versatility of the imaging techniques to image different types of polymeric MNs, two different polymers were used. To observe transdermal drug delivery in tissues using PAM, a solid MN was fabricated using poly(methyl methacrylate) (PMMA). The drug molecules to be delivered were then coated on the surface of the MNs. In the second study using μ OCT, hydrogel forming microneedles using poly(styrene)-block-poly(acrylic acid) (PS-*b*-PAA) and polystyrene (PS) was fabricated.

5.2.1.1 Poly(methyl methacrylate) (PMMA) Microneedle Array

All chemical reagents unless specifically mentioned were obtained from Sigma-Aldrich (Singapore) and used without any modification.

The MNs were designed by Micropoint Technologies Pte Ltd (Singapore) using poly(methyl methacrylate) (PMMA) polymer in a 10×10 array with height of 600 μ m and base width of 300 μ m. PMMA polymer was chosen for its high mechanical toughness and safety to withstand MNs compression when inserted into skin. Briefly, the fabrication methodology involved an injection molding process with an injection melt temperature of 293°C, mold temperature between 60 and 120°C, injection velocity of 150 mm/s and hold pressure of 48 MPa. The PMMA MNs were coated with 100nm AuNPs. Briefly, the surface of the PMMA MNs was thoroughly washed with acetone and blown dry with nitrogen gas. The MNs surface was then plasma treated using Harrick Plasma Cleaner PDC 32G. The MNs were dipped into 0.1 ml of concentrated AuNPs solution for several times ensuring that the solution covered the surface of the MNs and was dried in the oven at 80° C overnight. To evaluate the MNs skin insertion

ability, MNs were inserted into treated fresh porcine skin using an applicator and removed after 1 minute. The treated skin tissue was fixed and stained using Haematoxylin and Eosin (H&E) for histological analysis.

5.2.1.2 Poly(styrene)-block-poly(acrylic acid) (PS-*b*-PAA) Microneedle Array

All chemical reagents unless specifically mentioned were obtained from Sigma-Aldrich (Singapore) and used without any modification. Polystyrene-block-poly(acrylic acid) (PS-*b*-PAA) with number average molecular weights (M_n) of 26,000 g/mol for PS and 128,000 g/mol for PAA was purchased from Polymer Source Inc (USA). SeaKem® LE Agarose (Gelling temperature (1.5%): $36^{\circ}\text{C} \pm 1.5^{\circ}\text{C}$) was purchased from Lonza (Singapore). Microneedle (MN) stainless-steel master structure having a 10 x 10 array of conical cavities with a height of 600 μm and base width of 300 μm was purchased from Micropoint Technologies (Singapore).

The polymeric MN array used in the following imaging studies was prepared via a solvent casting method as previously reported.[147] Briefly, polydimethylsiloxane (PDMS) micromold was formed by pouring PDMS mixture consisting of 10:1 w/w ratio of pre-polymer to curing agent into the stainless-steel master structure before curing at 70°C for 2 hours and peeling it off gently from the micromold. The polymer casting solution was prepared by dissolving 100 mg of PS-*b*-PAA in 1mL of dimethylformamide (DMF) and solution mixing with Fe_3O_4 nanoparticles in different weight percentage (wt%) concentrations. To prepare the first layer of the MN patch, 100 μL of the polymer solution was poured into the micromold followed by centrifugation (3,134 $\times$ g, 5min) in a swinging bucket rotor (SCANSPEED 1580R, LaboGene) to force the polymer solution into the mold cavities. The solution was left to dry for 48 hours in the chemical hood at room temperature. The second layer of the MN was formed by melting polystyrene (PS)

pellets (Mw: 35k) on the top of the mold at 180 °C under vacuum for 4 hours. The MN patch was then gently peeled off from the mold after cooling to room temperature.

Fe₃O₄ nanoparticles were prepared by decomposition of ironpentacarbonyl Fe(CO)₅ in the presence of oleic acid (OA) followed by oxidation using trimethylamine N-oxide (CH₃)₃NO.[148] The hydrodynamic diameter and zeta potential of the nanoparticles was analysed with a Zetasizer Nano ZS (Malvern instruments).

The MN arrays were tested for its mechanical properties to ensure it has sufficient strength for skin penetration. To achieve this, MNs containing different amounts of Fe₃O₄ nanoparticles were examined using an Instron 5543 Tensile Meter. A vertical oriented force was applied perpendicularly to the MN tips facing upward on a flat-rigid aluminum-plate, using a 5-mm-diameter flat-head stainless steel cylindrical probe (at a constant speed of 0.5mm/min). The displacement was measured until a preset maximum force of 8N was achieved.

Next, to ensure the swellability of the MNs are not affected due to the presence of the Fe₃O₄ nanoparticles in the MN tips, swelling studies were conducted. Individual MN patches containing different amounts of Fe₃O₄ nanoparticles were weighed in the dry state (m_0) at the zero time point. The MN patches were then gently pressed onto 1.4wt% agarose gel prepared using PBS (pH 7.4). The MN patches were then removed at specific time points (i.e. 1min, 3min and 5min) and weighed in the swollen state (m_t). The percentage swelling ratio was determined as the following:

$$\% \text{ Swelling} = \left(\frac{m_t - m_0}{m_0} \right) \times 100\%.$$

To investigate the effect of Fe₃O₄ nanoparticles in μ OCT imaging of polymeric MNs, we studied the image contrast-to-noise ratio (CNR) for MNs with and without Fe₃O₄ nanoparticles. The CNR of an OCT image is defined as follows [149]: $CNR =$

$20 * \log \left(\frac{|S_A - S_B|}{\sigma_B} \right)$ where S_A is the average signal intensity of the area of interest (AOI), and S_B is the average signal intensity of background noise region (BNR) and σ_B is the standard deviation of the signal intensities in BNR.

All data were analysed using unpaired Student t-test, and the results were expressed as mean $\pm$ standard deviation. A p value < 0.05 was considered to be statistically significant in our study.

5.2.2 Optical Resolution Photoacoustic Microscopy (OR-PAM) System

The OR-PAM system (**Figure 5.1**) employs a nanosecond tunable laser system; consisting of a diodepumped solid-state Nd-YAG laser and a dye laser. The laser system can be tunable between 559 nm-576 nm. The laser beam was reshaped by an iris and then spatially filtered by a combination of condenser lens, CL (LA4327, Thorlabs) and a 50 μm pinhole, PH (P50S, Thorlabs). The attenuated beam through a variable neutral density filter, NDF (NDC-50C4M), was launched on to a single-mode fiber, SMF (P1-460B-FC-1, Thorlabs) using a fiber coupler, FC (F-91-C1, Newport).

Figure 5-1: Schematic illustration of the OR-PAM imaging system. BS - Beam Sampler, CL - Condenser lens, PH – Pinhole, NDF- Neutral density filter, PD – photodiode, FC – Fiber coupler, SMF - Single mode fiber, L1&L2 - Achromatic lens, M - Mirror, UST – Ultrasound transducer, DAQ - Data acquisition card, RA - Right angle prism, RP - Rhomboid prism, SO – Silicon oil, AL - Acoustic lens.

The beam out from the SMF was collimated by an Achromatic lens, L1 (32-317, Edmund Optics), reflected by a stationary elliptical mirror, M and filled the entire back aperture of another identical Achromatic lens, L2. The focusing beam will be passed through an optoacoustic beam combiner which consist of a right-angled prism, RA

(PS615, Thorlabs) and a rhomboid prism, RP (47-214, Edmund optics) with a layer of silicon oil, SO (DMPS1M, Sigma Aldrich) in between. The silicon oil layer acts as an acoustic reflective and optical transparent film. An acoustic lens, AL (LC4573, Thorlabs) was attached at the bottom of the rhomboid prism provided acoustic focal diameter $\sim 46 \mu\text{m}$. The ultrasonic transducer (UST), 50 MHz center frequency was placed on top of the rhomboid. The optical and acoustic foci were aligned confocally to maximize the detection sensitivity. The laser repetition rate for the OR-PAM was set to 5 kHz and the laser energy at focus can be varied up to 200 nJ per pulse. The laser wavelength was set at 570 nm.

The OR-PAM scanner head was attached to a 3-axis motorized stage connected to the computer. For photoacoustic imaging the bottom of the OR-PAM scanner head was submerged in a water-filled tank. For optical and acoustic transmission an imaging window (7 cm x 7 cm) was opened in the bottom of the tank and sealed with a polyethylene membrane. The PA signal acquired by the UST was amplified by two 24 dB amplifiers (ZFL-500LN, Mini Circuits), and was recorded in a desktop computer using a data acquisition card, DAQ. The scanning and data acquisition was controlled using Labview software. The synchronization of the data acquisition and the stage motion was controlled through the signal from a photodiode, PD. Two-dimensional continuous raster scanning of the imaging head was used during image acquisition. This OR-PAM system has a spatial resolution of ~ 4.2 micron, axial resolution of ~ 33 micron, and an imaging depth up to ~ 1.4 mm as characterized previously [117, 150].

For *in vivo* imaging, according to American National Standards Institute (ANSI) laser safety standards [151], the maximum pulse energy by a single laser pulse (MPE_{SP}) on the skin surface shouldn't exceed $\text{MPE}_{\text{SP}} = 2.C_A \times 10^{-2} \text{ J/cm}^2$, where C_A is the

wavelength correction factor, unity for visible wavelength range (400-700 nm). If a point on skin is exposed to more than 10 seconds the irradiance should not exceed 200 mW/cm². In the case of raster scanning, a point on the skin is not exposed for 10 seconds, hence the maximum permissible exposure (MPE_{AVG}) is limited by $1.1C_A t^{0.25}$ in mJ/cm² where t denotes the exposure duration in seconds. For OR-PAM, we believe due to optical aberrations at the prism surface and acoustic lens objective NA might have reduced from 0.11 to 0.075. Assuming the optical focus is 150 microns below the skin surface for *in vivo* imaging, the surface spot size was 22.5 μm in diameter and the calculated optical fluence for a single laser pulse was 20.4 mJ/cm² (90 nJ/pulse) close to the safety limit. Having a minimum pixel separation of 2 μm, an average of 11 (N) adjacent laser pulses overlaps on the skin surface. At 5kHz LRR the exposure time is 2.4 ms. So the MPE_{TRAIN} was 238 mJ/cm². The MPE_{SP} for the pulse train was (MPE_{AVG}) = MPE_{TRAIN} / N = 238 / 11 = 21.6 mJ/cm² close to the maximum permissible limit.

5.2.2.1 Imaging Methodology

The ear of a female mice of body weight 25 g and age 4 weeks, procured from InVivos Pte. Ltd. Singapore, were used for the imaging experiments. Animal experiments were performed according to the approved guidelines and regulations by the institutional Animal Care and Use committee of Nanyang Technological University, Singapore (Animal Protocol Number ARF-SBS/NIE-A0263). The animals were anesthetized using a cocktail of Ketamine (120 mg/kg) and Xylazine (16 mg/kg) injected intraperitoneally (dosage of 0.1 ml/10 gm). The imaging site was chosen to be at the ear of the mouse and thus the hair was removed before positioning the mouse on a microscopic glass slide. The MNs coated with AuNPs was pressed into the mouse ear using an applicator for 60 seconds and removed before OR-PAM imaging was performed. Mouse ear was used as a suitable model to mimic the human epidermis

thickness. In addition, the planar surface of the mouse ear tissue enables uniform pressure to be applied during MNs insertion to ensure fair distribution of AuNPs in the treated region for effective monitoring of AuNPs delivery. The animal was further anesthetized with vaporized isoflurane system (1 L/min oxygen and 0.75% isoflurane) during the imaging period. The imaging region was made in contact with the polyethylene membrane using ultrasound gel. To characterize the penetration depth as well as the rate of diffusion of AuNPs at each depth, a smaller region of interest ($1.5\text{ mm} \times 0.75\text{ mm}$) was scanned at different time intervals after MNs insertion with a step size of $2\text{ }\mu\text{m}$ along X-axis and $4\text{ }\mu\text{m}$ long Y-axis. A $2\text{ mm} \times 1.25\text{ mm}$ untreated area of the mouse ear was imaged as a control image with a step size of $2\text{ }\mu\text{m}$ along X-axis and $5\text{ }\mu\text{m}$ along Y-axis.

5.2.3 Micro-Optical Coherence Tomography (μ OCT) System

A lab-built μ OCT system (**Figure 5.2a**) was employed for MN imaging. Its laser source was a superluminescent diode array (Superlum Broadlighters T-850-HP) with a center wavelength of $\lambda_c = 850$ nm and a full width at half-maximum (FWHM) bandwidth of $\Delta\lambda = 165$ nm. The laser output power was ~ 20.3 mW. The light generated by the laser source was split into two paths by a broadband 50:50 fiber coupler (TW850R5A2, Thorlabs Inc.). The light directed to the reference arm passed through a polarization controller and a collimation lens L1 (AC050-015-B-ML, Thorlabs Inc.) first, and was then focused onto the reference mirror M1 by a lens L2 (AC254-030-B-ML, Thorlabs Inc.). The sample arm optics followed the same path length as the reference arm with a galvo scanner (GVSM002/M, Thorlabs Inc.) inserted in between the collimation lens L3 (identical to L1) and the objective lens L4 (identical to L2) for sample scanning.

Figure 5-2: Schematic of the μ OCT system. (a) The μ OCT system configuration. (b) The portable scanning head. (c) The tissue imaging configuration in experimental process. PC: polarization controller; SMF: single-mode-fiber; L1-5: achromatic lens; L6: camera lens; M1: reference mirror.

The illumination power on the sample was measured to be 6.98 mW. The collimation lens L3 together with the galvo scanner and the objective lens L4 constituted a compact scanning head (**Figure 5.2b**). Specifically, on such a scanning head, there was a portable window holder with a glass window placed below the objective lens. The glass window's back surface was fixed around 200 μ m above the focal plane such that once the MN patch was in direct contact with the tissue, the sample light would be approximately focused at the MNs. When the μ OCT was used for real-time MNs diffusion effect imaging, the tissue samples were placed on the glass window, while the MNs were placed on a motion stage such that they could be moved to penetrate through the tissues gradually (**Figure 5.2c**). It is worth noting that in this imaging process, another identical glass window was also inserted into the reference arm optics for dispersion compensation. While if the μ OCT was utilized for MN geometry characterization, both glass windows and the portable window holder were removed from the system.

The light backreflected from the reference arm and those backscattered from the sample arm were recombined by the fiber coupler to generate interferometric signals, which finally were guided to a spectrometer. The spectrometer was comprised of a collimation lens L5 (AC127-030-B-ML, Thorlabs Inc.), a diffraction grating (1200 l/mm @ 830nm, Wasatch Photonics Inc.), a camera lens (Nikon AF Nikkor 85mm f/1.8D), and a line scan CCD array (E2V, AViiVA EM4). The spectrometer efficiency, including the grating efficiency and the overall losses of optical components and spectrometer geometry, was measured to be 0.416. The detected signal was transferred to a computer through camera link cables and an image acquisition card (KBN-PCE-CL4-F, Bitflow Inc.) at 12-bit digital resolution. In the experiments, both the camera and the galvo scanners were synchronized by a triggering signal S-4 generated by the

computer. The effective camera pixel number covered by the laser spectrum was measured to be 1322 at FWHM.

Figure 5-3: μ OCT system spatial resolution characterization. (a) Transverse resolution was measured to be $\sim 5.5 \mu\text{m}$. (b) The axial resolution was measured to be $2.3 \mu\text{m}$ in air.

The spatial resolving power of the μ OCT system was characterized by measuring its lateral and axial resolutions respectively. A 1951 USAF resolution chart was imaged for system's lateral resolution measurement. An en face image of the resolution chart acquired, consists of $1024 \text{ pixels} \times 1024 \text{ pixels}$, covering an area of $0.52 \text{ mm} \times 0.52 \text{ mm}$ (**Figure 5.3a**). The line pattern of group 6 element 4 with a line spacing of $5.52 \mu\text{m}$ was resolved clearly, and therefore, the system lateral resolution was estimated to be $\sim 5.5 \mu\text{m}$, which matched well with the predicted $\sim 5.89 \mu\text{m}$. To measure the system's axial resolution, we inserted an actuated iris diaphragm (SM05D5, Thorlabs Inc.) with total attenuation of 37.2 dB into the sample arm optics and measured an A-line profile by placing a BK7 prism (with attenuation of 14 dB) at the focal plane of sample arm optics. The measured axial point spread function (PSF) indicates that an axial resolution of $\sim 2.3 \mu\text{m}$ was achieved in air, which is slightly larger than the theoretical value of $\Delta z = 1.93 \mu\text{m}$ (**Figure 5.3b**). Furthermore, as the signal-to-noise

ratio (SNR) was measured to be 52.4 dB at a path length difference of $\sim 110 \mu\text{m}$, the μOCT system sensitivity was estimated to be ~ 103.6 dB. Such a value was slightly lower than the theoretical value of 105.2 dB due to the imperfect responses of the optical components.

5.2.3.1 Imaging Methodology

The μOCT system was used to study the Fe_3O_4 PS-*b*-PAA /PS MNs' geometry by scanning the MN array over a certain area. The scanning range of the μOCT system was set such that the whole MN array was covered; for several B-scan frames to be obtained sequentially. In this study, a B-scan frame typically consists of 1024 pixels (W) $\times$ 1100 pixels (H), covering an area of 9.6 mm (W) $\times$ 0.7 mm (H). The obtained B-scan frames were finally combined to reconstruct 3D images of the MN patch consisting of 1024 pixels (L) $\times$ 1024 pixels (W) $\times$ 1100 pixels (H), covering a volume of 9.6 mm (L) $\times$ 9.6 mm (W) $\times$ 0.7 mm (H).

To perform a quantitative study on the swelling effect of the MNs, both cross-sectional and three-dimensional (3D) images of the MN patch were recorded with the μOCT system before and after its insertion into three different mediums; 1.4wt% agarose hydrogel, rat ear skin and human skin samples. Each experiment was repeated at least 3 times with the same μOCT setup to avoid artifacts caused by the μOCT system. The skin samples used in the study were freshly explanted with minimal treatment for imaging. The following study protocols were reviewed and approved by both institutional animal care and use committee (IACUC) and institutional review boards (IRB) at Nanyang Technological University (NTU) [ARF-SBS/NIE-A0312, ARF-SBS/NIE-A0302].

To observe the swelling effect of the MNs in 1.4wt% agarose hydrogel, the μ OCT system was utilized to scan the MN array repeatedly at the same location for 1-2 minutes following MN insertion. The scanning range of the μ OCT is sufficient to cover the width of the MN array, and thus several cross-sectional images of a row of MNs could be obtained sequentially. The measured widths of the swollen MNs in the cross-sectional images could clearly illustrate the swelling effect of the MNs in a time-dependent manner.

Similarly, the same procedure was followed to study the swelling behaviour of the MNs in mouse ear. B-scan imaging of a row of MNs were conducted from the time when the MNs were inserted into the sample for ~ 2 minutes till the MN patch was pulled out. Specifically, it is worth mentioning that for MNs' swelling effect imaging in both hydrogel and skin tissues, a skin gel (Aquasonic 100, Parker Laboratories Inc.) was applied in between the glass window and the samples to prevent strong reflections caused by the refractive index mismatching.

To illustrate the swelling effect of the MNs in human skin, both B-scan and 3D imaging were performed. Those two processes were conducted in two separate steps. For the 3D imaging, the skin tissue was placed on top of the glass window of the portable μ OCT scanning head, and imaging was done before and after MNs' penetration. For the B-scan imaging, the MNs were scanned over the same location following insertion into the skin tissue, to obtain several cross-sectional images of the MNs. The imaging process took approximately 5 minutes to allow the MNs to reach their maximum swellability. In this study, the system scanning speed was set to be 20,480 A-lines/s with each frame consisting of 1024 A-lines.

5.3 Results and Discussion

5.3.1 In vivo studies of Transdermal Drug Delivery with Microneedles using OR-PAM

The fabricated MNs array has a dimension of 1 cm by 1 cm with solid MNs tips of height 600 μm and base width of 350 μm (**Figure 5.4a and 5.4b**). The PMMA MNs could easily penetrate the fresh porcine skin tissue to a depth of 400 μm as observed in the H&E staining, in which MNs tip reached the dermis layer (**Figure 5.4c**). The difference observed between the needle length and the actual penetration depth (displacement) is a result of skin elasticity [152]. The observed penetration depth is adequate for effective intradermal drug delivery in human skin.

Figure 5-4: Characterization of PMMA MN array. (a) Camera image of the PMMA MNs (b) Optical microscope image of the MN array (c) H&E staining of PMMA MNs treated porcine skin showing the depth of MN penetration.

To illustrate the use of PAM imaging in the study of MN mediated transdermal drug delivery, AuNPs is used as a model drug and coated on PMMA MN array. The AuNPs were coated on PMMA MNs via facile dip-coating. The coating of AuNPs on MN surface did not result in any significant change in the MN morphology (**Figure 5.5a**). At a greater magnification, clusters of AuNPs can be observed on the pyramidal

sides of the MNs tips (**Figure 5.5b**). Taking together, the dip-coating process successfully coated PMMA MNs with AuNPs without influencing the structural integrity of the MNs.

Figure 5-5: SEM characterization of AuNPs coated PMMA MN array. (a) A top view; (b) Zoom-in visualization of the AuNPs on a coated PMMA MN.

To study the penetration effects of MNs, firstly a control image of the blood microvasculature in the mouse ear skin before the MNs treatment was obtained under the Maximum Amplitude Projection (MAP) OR-PAM (**Figure 5.6a**). Following the insertion of the AuNPs coated MNs, certain areas of the microvasculature (**white dotted box, Figure 5.6b**) were observed to have nanoparticle deposition in a patterned way similar to the arrangement of the MNs tips in the designed MN array. As AuNPs have a strong optical absorption (resulting in strong photoacoustic signal) visualizing and monitoring the delivery of these particles in the skin after its detachment from the MNs is easily achieved. This results concludes the successful MNs penetration and delivery of the AuNPs using MNs. In addition, it is also notable that no visible damage is observed to the blood vessels structure following the MNs intervention.

Figure 5-6: In vivo photoacoustic imaging of mouse ear (a) before and (b) after the AuNPs coated MNs insertion.

Next, MAP photoacoustic images at different time intervals (after the MNs insertion) of 5 minutes, 40 minutes, 95 minutes, and 145 minutes were obtained respectively (**Figures 5.7a-d**). Using these images, the real-time diffusion process of the AuNPs in the tissue can be observed. With time progression, a lesser photoacoustic (PA) signal intensity is recorded indicating a decreasing diffusion flux. To quantify the penetration depth of the AuNPs, Figure 5.7a was segmented into image stacks in increasing depth from the skin surface and represented as Figure 5.7e. Using these images, we can clearly visualize the accumulation of the nanoparticles along the z- axis of the tissue in different concentrations. Up to a depth of 150 μm in mice ear tissue, we confirm the deposition of the nanoparticles using MNs delivery.

To quantify the observations from the MAP images, the PA signal amplitude is used as a suitable parameter. A reduction in the PA signal amplitude with time at the sites of AuNPs accumulation can be interpreted as decreasing rate of diffusion. The rate of diffusion of AuNPs with time and along the depth profile was measured using the average PA signal amplitude of a small region of interest ($0.4\text{ mm} \times 0.4\text{ mm}$ shown in white dotted area) at different time intervals and at different depths.

Figure 5-7: *In vivo* photoacoustic microscopy (Maximum amplitude projection) images of mouse ear. Data acquisition started (a) 5 min, (b) 40 min, (c) 95 min, and (d) 150 min after AuNPs coated microneedle insertion. (e) Image slices of image (a) at various depths. (f) Variation of photoacoustic signal amplitude over time at various depths.

The plot of average PA signal amplitude with time and depth (**Figure 5.7f**) shows that the maximum PA amplitude was attained immediately after MNs insertion at each of the measured depths and reduced significantly with time. In addition, the rate of diffusion was significant in the first 90 minutes following MNs insertion and no significant decrease in the PA amplitude was observed thereafter indicating a plateau in the diffusion effect.

These results can be interpreted using Fick's First Law of Diffusion; which states that particles diffuse with a flux proportional to the concentration gradient. As time

progressed, the AuNPs diffused laterally in a steady state and hence at each of the measured depth, we observe a similar trend in the PA signal. The PA amplitude signal measured at the surface of the tissue is comparable to the signal attained at a depth of 180 μm . This can be explained by the limitation of the sampled tissue as the mice ear has a thickness of 150-170 μm .

Substantially, AuNPs are capable of enhancing singlet oxygen generation and have shown to improve the efficacy of conventional photodynamic therapy [153, 154]. Hence, AuNPs can be used as potential therapeutic agents to destroy tumor cells [155, 156]. As it can be interpreted that the diffusion process of AuNPs is relatively quick and takes only approximately 20 minutes inside the tissue, this study proposes the possibility of using transdermal delivery of AuNPs through polymeric MNs as a treatment alternative in photodynamic therapy [157].

5.3.2 In vivo studies of Skin Interstitial Fluid Extraction with Microneedles using μ OCT

The swellable Fe_3O_4 MN arrays were prepared using polystyrene-block-poly (acrylic acid) (PS-*b*-PAA) and polystyrene (PS) polymers via a solvent casting method (**Figure 5.8**). PS-*b*-PAA polymer was chosen to fabricate the tips of the MN array as it contains carboxylic acid groups which become ionized in the presence of water enabling the extraction of fluid when being placed within a tissue [109]. PS polymer was used as the base material of the MN structure to provide mechanical strength and structural integrity required to penetrate tissues without deformation.

Figure 5-8: Schematic illustration of the fabrication process of the double-layered Fe_3O_4 PS-*b*-PAA/PS MN patches. Fe_3O_4 nanoparticles were localized on the MN tips of the needles.

Specifically, a negative polydimethylsiloxane (PDMS) mold from a stainless-steel MN template was fabricated first (**Figures 5.8a and 5.9b**). Fe_3O_4 nanoparticles with a diameter of 40 nm and zeta potential of +18.2 mV were mixed with 100 mg/ml of PS-*b*-PAA in different weight percentages(wt%) using dimethylformamide (DMF) as the solvent. The nanoparticle-polymer mixture was stirred vigorously for 24 hours

before it was placed in a vacuum oven to degas for 2 hours and casted onto the PDMS negative mold. After being dried for 48 hours in a fume hood, a thick film was formed in the tip region of the mold due to the presence of capillary forces (**Figure 5.8c**). The PS pellet was then melted on top of the thin PS-*b*-PAA film remaining in the mold at 180°C under vacuum for 4 hours to produce the double layered needle structure (**Figures 5.8d and 5.9e**). The designed MNs have a height of approximately 700µm with a base width of about 300µm and an interneedle spacing of 400µm.

To observe the swellability of the PS-*b*-PAA/PS MNs, conventional microscopy was first used to visualize the changes in the MN morphology after fluid absorption. The MN patch was inserted into a 1.4wt% agarose gel covered with a thin strip of Parafilm M to prevent direct contact of the MN tips with the surface of the agarose gel. After an application duration on 1 minute, the MN array was carefully removed and placed under microscope to visualize the MN tips. In comparison with the image taken before insertion into the agarose gel, the MN tips appeared round-shaped and swollen indicating the presence of fluid in the MN matrix (**Figures 5.9b and 5.9c**). Unfortunately, these images were not sufficient in determining the accurate quantifications of the fluid volumes extracted. Additionally, to evaluate the maximum swellability of the PS-*b*-PAA/PS MNs, different application durations were tested out on the agarose gel. But the lack of a real-time *in situ* visualization of the swelling process limited the ability to determine the optimum duration for achieving maximum fluid absorption.

Therefore, to monitor the real-time swelling characteristics of MNs non-invasively and quantitatively analyze their swellability in biological tissues, a lab-built µOCT system is adopted. The contrast enhancement in µOCT imaging of polymeric MNs is shown to be provided by the encapsulation of Fe₃O₄ nanoparticles in the MN

matrix. Following, the preparation of Fe_3O_4 -PS-*b*-PAA/PS MNs, optical microscopy was also used to observe the change in the MN morphology after insertion into 1.4wt% agarose gel. The addition of Fe_3O_4 nanoparticles resulted in a color change from transparent to brown. Similarly, the tips attained a swollen morphology after coming into contact with fluid but did not allow the exact quantification of fluid volume to be determined (**Figures 5.9e and 5.9f**).

*Figure 5-9: PS-*b*-PAA/PS MN patches with and without Fe_3O_4 nanoparticles. (a) and (b) PS-*b*-PAA/PS MN patch. (c) PS-*b*-PAA/PS MN patch after immersion in the agarose hydrogel for 1 minute. (d) and (e) Fe_3O_4 PS-*b*-PAA/PS MN patch. (f) Fe_3O_4 PS-*b*-PAA/PS MN patch after immersion in the agarose hydrogel for 1 minute. (Scale bar: 1 mm)*

Although Fe_3O_4 nanoparticles may provide a significant contrast enhancement for real time monitoring of swellability of polymeric MNs, the addition of Fe_3O_4 nanoparticles should not significantly alter the properties of the PS-*b*-PAA/PS MNs. Hence, the concentration of Fe_3O_4 nanoparticles encapsulated in the MN array for μOCT imaging was optimized to retain the mechanical strength and swellability. Firstly, the loading concentration of Fe_3O_4 nanoparticles was tuned between 2 and 8wt % in the PS-*b*-PAA/PS MNs. The mechanical strength of the MNs was studied with an axial compression test, in which the MNs were compressed and the deformation at various loads was recorded. Compressive stress and strain were calculated and plotted as a stress–strain diagram (**Figure 5.10a**). With a concentration of 5wt% or less of Fe_3O_4 nanoparticles, there was no significant change in the mechanical strength of the MNs. However, exceeding this concentration it was observed that the MN tips bend easily when being peeled off from the PDMS mold. This observed phenomenon is mostly because the entire tip structure of the MN is filled with the inorganic nanoparticles causing it to become very soft. The increasing Fe_3O_4 concentration in the PS-*b*-PAA polymer could possibly alter the extent of crosslinking between the PS block in the PS-*b*-PAA layer and the PS homopolymer layer at the molecular level. It is also observed that the double-layered structure is less prevalent with increasing nanoparticle concentration as the PS base layer occupies less cavity space in the MN mold. Therefore, the loading concentration should be controlled between 2 and 5wt % to retain the mechanical strength of the PS-*b*-PAA/PS MNs.

Next, the swellability of the MNs was evaluated by inserting MNs with different concentrations of Fe_3O_4 nanoparticles into 1.4 wt% agarose hydrogel at room temperature for 1, 3, and 5 min. A thin strip of Parafilm M was used to cover the surface of the agarose hydrogel. The film mimics the stratum corneum barrier and prevents the

direct contact of the MN base with the hydrogel. The swellability of the MNs was quantified by weighing the MN patches before and after insertion into the hydrogel. The mass increase was computed as a percentage swelling ratio and compared against that of blank PS-*b*-PAA/PS MNs. It was observed that a longer immersion time in agarose resulted in a higher swelling ratio, and such a trend was observed consistently in MNs encapsulated with different concentrations of Fe₃O₄ (**Figure 3.10b**). However, the encapsulation of Fe₃O₄ nanoparticles did not significantly influence the swelling ratio regardless of the concentration used. This could be due to the relatively smaller size of the nanoparticles in comparison with the micrometer-sized MN tips. It also could be that the encapsulation of Fe₃O₄ nanoparticles did not influence the chemical properties of the PS-*b*-PAA/PS MN resulting in the preservation of its swellability behaviour.

The use of Fe₃O₄ nanoparticles for contrast enhancement in μ OCT imaging was studied by measuring the contrast-to-noise ratios (CNRs) of the OCT images of Fe₃O₄-PS-*b*-PAA/PS MNs (**Figure 3.10c**). MN patches with different concentrations of Fe₃O₄ nanoparticles were prepared and utilized for μ OCT imaging. The cross-sectional images of a row of MNs were acquired and averaged (eight images were utilized) while the contrast of such obtained images was adjusted to its best values for CNR comparison. The CNR improved with an increasing Fe₃O₄ concentration until it reached a plateau at

4wt %. This should be due to the saturated light-backscattering effect with a concentration of Fe_3O_4 nanoparticles higher than 4wt %.

Figure 5-10: Characterization of Fe_3O_4 -PS-*b*-PAA/PS MNs. (a) Mechanical strength (b) Swellability and (c) CNR of Fe_3O_4 -PS-*b*-PAA/PS MNs with different weight percentage loading concentrations of Fe_3O_4 nanoparticles.

It is critical that the encapsulation of nanoparticles affect minimally or do not affect the mechanical properties and swellability of PS-*b*-PAA MNs. Thus, to achieve a compromise between the CNR enhancement and the mechanical strength of Fe_3O_4 -PS-*b*-PAA/PS MNs, a 5wt% Fe_3O_4 nanoparticle loading concentration was chosen as a suitable contrast agent for μOCT imaging of polymeric MNs.

The encapsulation of Fe_3O_4 nanoparticles in the MNs allows for clear and distinct visualization of the tomography of PS-*b*-PAA/PS MNs with μOCT imaging. To compare the contrast effect due to the presence of Fe_3O_4 nanoparticles both cross-sectional images and 3D reconstruction images of the PS-*b*-PAA/PS MNs with and without Fe_3O_4 nanoparticles were obtained. Without the presence of Fe_3O_4 nanoparticles it is difficult to distinguish the MNs from the background (**Figures 5.11a and 5.11b**). Similarly, when the MNs were inserted into the hydrogel, the backscattered light signal from the MNs was significantly lower than that from the background (i.e., hydrogel) making the visualization of the insertion and swelling of the MNs in hydrogel

impossible (**Figure 5.11c and 5.11d**). In contrast, the presence of Fe_3O_4 nanoparticles significantly improved the μOCT contrast by increasing the backscattered light signal from the surface of the polymeric MNs. The morphology of the MNs was distinct in both 2D and 3D images (**Figures 5.11e and 5.11f**). Following the insertion of the MNs into the hydrogel, they still presented identifiable OCT signals (**Figure 5.11g and 5.11h**).

*Figure 5-11: Contrast enhancement of Fe_3O_4 nanoparticles in μOCT imaging of PS-*b*-PAA/PS MNs. (a–d) 2D and 3D images of PS-*b*-PAA/PS MNs; (e–h) 2D and 3D images of Fe_3O_4 PS-*b*-PAA/PS MNs in air and agarose hydrogel; and (i) calculated CNRs for a–h using the OCT signals in AOIs and BNRs in the corresponding images. (Scale bars:*

vertical-0.1 mm and horizontal-1 mm; yellow arrows: MN base; and green arrows: MNs)

To have a better understanding of the contrast difference aided by the presence of Fe_3O_4 nanoparticles, the CNR of the images obtained was measured and compared. The area of interests (AOIs, i.e., the MNs) and background noise regions (BNRs, i.e., the background) were marked with yellow dashed boxes (**Figure 5.11a and 5.11e**). In both air and hydrogel, the calculated CNR of the OCT images for Fe_3O_4 PS-*b*-PAA/PS MNs is more than 20 dB (i.e., at least 10 times) higher than that of PS-*b*-PAA/PS MNs (**Figure 5.11i**) indicating the improved image resolution observed.

The contrast enhancement of μOCT imaging of MNs under the static condition motivates to further explore whether this would allow the real-time imaging of MN based ISF extraction. Fe_3O_4 PS-*b*-PAA/PS MN array was inserted into 1.4wt% agarose and the scanning beam was focused onto a row of MNs to acquire time series B-mode frames for 45 seconds (**Figure 5.12a**). To quantitatively evaluate the change in the morphology of the MNs in in vitro conditions, each MN was modelled to be a circular cone to calculate the average volume of the MNs within the agarose at different times. The swelling behaviour of the MNs was analysed by computing the average MN volume changing ratio, defined as the ratio of the average MN volume in the agarose gel at different times to that at 1 second.

Following the insertion of the MNs into agarose it is observed that the increase in the amount of fluid absorbed was minimal during the first 3 seconds. When the MNs started to penetrate completely into the hydrogel the swelling process was fast. In 7 seconds, the volume of the MNs increased by $\sim 200\%$. Ten seconds later, the polymer

matrix was saturated with water, and thus, there was only a slight $\sim 28\%$ increase in the volume in the next 30s (**Figure 5.12b**).

*Figure 5-12: Real-time μ OCT imaging of insertion of PS-*b*-PAA/PS MNs into the agarose hydrogel. (a) Time-lapsed cross-sectional views of MNs during the insertion process and (b) quantification of swelling kinetics of MNs at different time points. (Scale bars: vertical-0.1 mm and horizontal-0.5 mm)*

The swelling behaviour of the MNs in fresh mouse ear skin tissues was also examined using the same methodology. When imaging, a small piece of skin tissue was placed onto the glass window on the portable OCT scanning head while the MN patch was placed on a motion stage and moved gently towards the skin tissue to replicate penetration. It took approximately 15 seconds for the insertion process, and after 50s the MNs were pulled out from the skin (**Figure 5.13a**). Similar to the analysis of insertion and swelling of MNs in agarose, the swelling process of the MNs in the mouse skin was

also quantified by MN volume changing ratio over time (**Figure 5.13b**). During the first 10 seconds of the insertion process, the change in the morphology of the MNs was not apparent because the skin was not completely punctured due to the existence of the stratum corneum layer and its skin elasticity. However, once the stratum corneum layer was punctured and the MNs were inside of the skin (10s onward), the MNs started to swell for about 30 s until the volume reached a maximum.

*Figure 5-13: Real-time μ OCT imaging of swelling kinetics of PS-*b*-PAA/PS MNs in mouse ear skin tissues. (a) Time-lapsed cross-sectional views of MNs during the swelling process and (b) averaged volume changing ratio per MN measured at different time points. (Scale bars: vertical-0.1 mm and horizontal-0.5 mm; yellow arrows: skin stratum corneum; and green arrows: MNs)*

The swelling process of MNs in skin (~ 30 s) is observed to be much slower as compared with that in agarose (~ 18 s). This can be explained by the lower water content in skin ($\sim 60\%$) than that in hydrogel ($>99\%$). It is worth mentioning that within the

whole process of insertion of MNs into the skin and pulling out from the skin, the real-time morphology changes in the profile of the inserted MNs could be observed clearly. This is because the addition of Fe_3O_4 nanoparticles increases the light backscattering effect from the MNs significantly improving the contrast between the MNs and the skin.

This methodology was finally extended to test its applicability in imaging MN swelling in human skin tissue. Before the insertion and imaging of MNs, the fat tissue was removed and then the processed skin tissue was placed on the top of the glass window of the portable OCT scanning head. The MN patch was placed on a motion stage and moved to penetrate the skin tissue; with the entire process monitored for ~5 minutes. The MN penetration into skin took about 15 s and the MNs were left in the human skin tissue for a total of 251 seconds (**Figure 5.14a**).

*Figure 5-14: Real-time μ OCT imaging of swelling kinetics of PS-*b*-PAA/PS MNs in the human skin. (a) Time-lapsed cross-sectional views of MNs during the swelling process, (b) averaged volume changing ratio per MN measured at different time points, (c & d) 3D and en face images of the skin tissue before the insertion of MNs, (e & f) 3D and en face images of the skin tissue after the insertion of MNs. (Scale bars: vertical-0.1 mm and horizontal-0.5 mm; yellow arrows: skin tissue; green arrows: MNs; and black arrows: glass surface)*

Similarly, the swelling of the MNs in human skin was also characterized by their volume changing ratio (**Figure 3.14b**). The swelling process in the human skin lasted for around 35 seconds which was slightly longer than that in the mouse ear skin. This suggests that compared to mice skin, a longer application time of the MN array is necessary when applied on human skin due to the presence of a thicker stratum corneum layer. From 40 to 75s, the average volume of the MNs remained approximately constant indicating maximum swellability was attained

Using the developed μ OCT system with enhanced contrast provided by Fe_3O_4 nanoparticles, the differences in the skin structure before and after MN penetration was imaged. The 3D and en face images were obtained at an imaging depth of 120 μm before and after the insertion of MNs into the skin tissue (**Figure 5.14c–f**). The observation of black holes in the skin after MN treatment illustrates the presence of microconduits left behind following MN penetration (**Figure 5.9f**). These images demonstrate that the MNs can achieve significant penetration in biological tissues to reach the viable epidermis where ISF collection is targeted.

5.4 Conclusion

With the use of MNs has a minimally invasive method of circumventing the barrier properties of the stratum corneum, the development of novel MN arrays is receiving much attention. Hence, the development of accurate characterization methods for designing and optimizing polymeric MN arrays would be of great benefit potential. In this chapter, the use of imaging technologies for MN characterization has been shown to be a valuable tool in the study of MN penetration and in-skin behaviours.

For the first time, the use of OR-PAM to study non-invasively MNs skin penetration and delivery of AuNPs *in vivo* using a mice model is investigated. PMMA MNs were able to efficiently penetrate skin tissue and deliver the AuNPs up to a depth of $\sim 150\ \mu\text{m}$. The diffusion pattern of delivered AuNPs in the skin tissue was observed and found to take ~ 90 minutes post the MNs insertion. This work reveals the potential use of photoacoustic microscopy in the *in vivo* monitoring of transdermal nanoparticle delivery using MNs platforms. Furthermore, with photoacoustic imaging finding its potential towards clinical translations [158] and the feasibility of making portable handheld devices; PAM makes for a promising imaging tool [159-161].

While OCT has been shown to be useful in MN imaging, it often lacks resolution necessitating post imaging processing. In this study, the use of Fe_3O_4 nanoparticles as suitable contrast agents for real-time high-resolution μOCT imaging of polymeric MNs has been demonstrated. With finely tuned concentrations, the presence of Fe_3O_4 nanoparticles in the MNs significantly improves the contrast in μOCT imaging by more than 10 times in both air and hydrogel and enables the observation of changes in MN profile without any significant alteration to the properties of the swellable MNs. As a proof of concept, the real time penetration and *in situ* monitoring of the swelling of PS-

b-PAA/PS MNs in different biological samples (hydrogel, mouse and human skin) was examined. The use of Fe₃O₄ nanoparticles plays a crucial role in contrast enhancement of polymeric MNs allowing non-invasive and quantitative evaluation of MN swelling behaviour. For the first time, this methodology realizes the real-time non-invasive observation and analysis of polymeric MNs' performance in transdermal fluid extraction.

The described techniques is anticipated to greatly facilitate researchers in the development of novel MN platforms for both transdermal drug and ISF extraction as greater ease and accuracy is achievable in studying key parameters such as penetration depth of MNs, drug release profile and skin recovery rate. Both the imaging studies performed above is relevant in the understanding of *in vivo* MN behaviours and it orchestrates the design and fabrication of more efficient polymeric MN-based drug delivery devices.

Chapter 6:

Summary and Future Outlook

6.1 Summary

MNs have been actively investigated as a minimally invasive technique of physical enhancement for transdermal drug delivery. In the last two decades, this technique has progressed in various modifications such as hollow, coated, dissolving and hydrogel forming MNs. Depending on the type of MN used, drugs are delivered either within the skin or deeper to the underlying tissues with a subsequent local accumulation effect or further release for systemic circulation. The predominant use of MNs was to provide microporation of the SC barrier to allow delivery of various drugs and vaccines. Yet, lately there has been an increasing focus on the use of MNs to access ISF for development of MN-based diagnostic sensors.

Different materials such as silicon, glass, metal and polymers have been widely used in the design of MN arrays depending on its desired applications. Silicon and glass have been attractive options because of well-established fabrication techniques but are relatively expensive materials compared to metals. Metals, on the other hand are brittle and suffer from poor biocompatibility and safety in case of accidental breakage in skin. In comparison, polymers have greater mechanical advantages owing to its viscoelastic property that results in improved resistance to shear-induced breakage. Most polymers used in the fabrication of MNs are low cost, display ease of manufacture and most importantly superior biocompatibility and biodegradability properties. Furthermore, depending on the inherent physicochemical properties of polymers MNs with various attributes such as dissolvable, swellable, and biodegradable can be designed that inevitably contributes to unique drug release profiles. Thus, the unification of the various features of polymers enables it to be an attractive material candidate in the development of novel MN arrays.

MNs have been widely used in transdermal drug delivery of many different types of molecules. Most of the MN technologies rely on passive diffusion to drive drug penetration from the epidermis towards deeper skin layers. This characteristic non-directional drug diffusion is driven by the concentration gradient and over time results in a decreasing diffusion rate. This can delay the onset of drug effect and result in lack of therapeutically relevant dose concentration. Alternatively, to achieve active and directional transdermal drug delivery, the development of a novel conductive MN array that is compatible with iontophoresis has been demonstrated. In the fabrication of the novel MN array, a biocompatible and conductive polymer poly(3,4-ethylenedioxythiophene)-poly(styrenesulfonate) (PEDOT:PSS) and biodegradable polymer hyaluronic acid (HA) is investigated. By using a combination therapy of both MNs and iontophoresis, a synergistic effect is observed in the acceleration of drug diffusion process. The results showed a significant increase in drug penetration depth in both *in vitro* and *in vivo* studies. This new technology opens up the potential of using different polymers in MN array design to bring about different characteristic behaviours; proving that a combination of several polymers can result in MNs with “smart” behaviour. Furthermore, the use of biocompatible polymers eases the clinical translation of such developments and results in patient-friendly devices.

The use of polymers in the design of hydrogel-forming MNs has also attracted vast attraction in the development of MN-based glucose sensors. Different types of MN designs such as hollow MNs and swellable MNs have been studied for sampling of ISF as a painless and minimally invasive alternative to blood sampling for glucose. The micrometre dimensions of MNs allows sufficient penetration depth to target the ISF present in the viable epidermis and upper layers of dermis without contacting the underlying nerves. However, the long administration time and offline analysis of current

MN sensors often compromises patient compliance and cost; limiting its use as a point-of-care system. To address the limitations of current swellable MNs, the use of a porous polymer that allows fluid flow from one side of the MN to the other is investigated for ISF extraction. Simultaneously, the integration of an enzyme based colorimetric glucose sensor to allow for rapid and real time analysis of glucose concentration in the extracted ISF in a single step is presented. Given the recent trend of easy-to-use point-of-care microdevices for personal healthcare monitoring, this proof-of-concept study opens a new avenue for the development of MN-based micro-devices for the application of sampling ISF and minimally invasive metabolites detection. The development of such a device can significantly improve treatment quality by offering a complete temporal representation of the glucose levels and at the same time reducing patient discomfort and complications of finger pricks in conventional monitoring in blood samples.

Leveraging on the promising use of polymers in the design and fabrication of MNs development of novel MN arrays has been carefully studied and elaborated in this thesis to overcome drawbacks of existing MN technologies. In the design of MN array, it is of paramount importance that there are appropriate techniques available to determine the influence of MN geometry on MN penetration depth. Currently, this is achieved by taking a biopsy of the MN-treated skin followed by traditional histological sectioning and staining. Such techniques are cumbersome, time-consuming and destructive in nature. Additionally, the freezing of treated tissue samples results in an altered skin structure changing the actual dimensions of the micropore created as a consequence of dehydration and mechanical insult.

While these methods are gold-standards used to provide conclusive information, being invasive and disruptive in nature meant that each sample only provides a snapshot of the dynamic processes such as dissolution and swellability of MN arrays. In turn,

multiple sample preparations and sacrifices would have to be made, should one intend to obtain time-dependent information of the processes.

To facilitate and drive the development of polymeric MN research, the successful use of μ OCT and OR-PAM is demonstrated as non-invasive techniques to visualize polymeric MN arrays in real time while they are in place in tissue. Of vital importance, μ OCT can be used to successfully follow *in situ* swellability of MNs in real time. Previously, determination of the swellability kinetics of hydrogel-forming polymeric MN require the MN to be removed from the skin, at a variety of time intervals, and the dimensional changes observed via light microscopy. Aside from the need for a large number of replicate experiments using this method, it could potentially lead to an inaccurate estimation of the true swelling ratio.

To date, OCT has been used to study MN penetration depth and to visualize the dissolution kinetics of dissolving polymeric arrays. However, post-imaging processing such as addition of false colors is performed to differentiate skin tissue and MNs. When μ OCT imaging was carried out with Fe_3O_4 nanoparticles as contrast agents, the need for additional post-processing is removed. Herein, the presence of Fe_3O_4 nanoparticles in the polymer matrix acts as a medium to enhance the backscattering of light resulting in high-resolution images. With finely tuned concentrations the *in situ* changes in MN profile during the swelling process is observed clearly when PS-*b*-PAA/PS MNs are inserted into agarose hydrogel, mice skin tissue, and human skin tissue. In addition, it was conclusive that presence of higher water content in agarose led to the quicker swelling process (i.e. 20s) whereas longer time was required for optimal swelling to occur in human skin tissue. Such findings is crucial in driving the development of polymer MN-based ISF extraction.

Likewise, the successful use of OR-PAM proves to be a key development for polymeric MN research. OR-PAM is a fast-growing hybrid *in vivo* imaging modality combining optical contrast with ultrasound resolution. It has been shown that OR-PAM is capable of non-invasive and quantitative characterization of MN penetration depth and delivery of AuNPs in a mice model. In addition, it allows the spatial distribution of the delivered AuNPs in the skin tissue to be observed in real time facilitating the study of coated polymeric MNs based drug delivery. To date, there has been no other method that can non-invasively visualise the real time, *in vivo* rate of drug diffusion following MN insertion. Furthermore, this is the first study to follow the spatial distribution of drug particles in skin in real time. As such, this technique opens up the possibility to investigate the consistency of MN penetration, drug distribution, and also skin recovery upon MN penetration.

Considering the growing needs of the MN field to develop MN platforms for successful transdermal drug delivery and biosensing; this particular work broadens the availability of tools for researchers to employ in the evaluation of the properties of MN-based drug delivery and ISF extraction in real time and *in-vivo*. This will be necessary if MN-based delivery systems are to achieve their massive potential and move forward from laboratory benchtops and animal studies to commercial clinical reality.

6.2 Future Directions

As hypothesized and briefly demonstrated herein, in the development of polymer-based MN technologies, it is necessary that non-invasive characterization tools are available to track development progression leading to an optimal design. The successful use of μ OCT and OR-PAM can benefit the translation of MN technologies in various ways, both for in vitro optimization as well as allowing more comprehensive pre-clinical studies to be performed. Several ways in which both the described techniques utilization could be adapted for other MN characterization are listed below.

As mentioned previously, MN swelling behaviour in human skin differ significantly from in vitro conditions such as agarose gel due to the different water composition present. Using μ OCT, the distinct real time swelling kinetics is observed allowing researchers to accurately determine MN application duration required for adequate fluid extraction. As such, this imaging technology can be extended for use in different drug delivery systems by choosing an appropriate wavelength of the laser to be used depending on the absorbance wavelength of the drug to be delivered. If necessary, drug carriers such as carbon nanotubes and porphyrins which can provide strong PA signals can be used to assess drug delivery profiles of small molecule drugs by MNs. In a similar way, it can be employed to study real time drug release profile of soluble/dissolvable MNs used in drug delivery applications. In the case of dissolvable MNs, it is critical to gain an understanding of the MN penetration depth to determine the fraction of the MN that resides within the skin following insertion. This is because the effective drug delivery rate will be dependent on the dissolution rate of the MN fraction that is in contact with the aqueous tissue environment.

Hence, to enable appropriate designing of an optimal polymer MN-based drug delivery system, it is important to visualize the in situ penetration depth and dissolution in real time to determine effective drug permeability. Most studies that assess MN penetration depth rely on the measurement of transepidermal water loss as a result of tissue destruction or simply an *en face* observation of the micropores on skin surface. The use of Fe₃O₄ nanoparticles as contrast agents in μ OCT imaging allows for an accurate measurement of the penetration depth following MN insertion into skin. Additionally, researchers can clearly visualize the dissolution profile of the MNs in real time gaining insights to the drug delivery process. This paves way for researchers to choose suitable polymers (i.e. different polymers have differing rates of dissolution) for the desired drug delivery outcome.

Following the successful use of OR-PAM system to observe the delivery of AuNPs using coated PMMA MNs, this method can be extended to study the delivery profile of different drugs using different polymeric MNs. Most MN-based drug delivery systems focus on the delivery of small molecule drugs and hydrophilic proteins. As rate of drug diffusion is size-dependant, it is important that a suitable model drug is used in drug delivery studies. AuNPs are suitable candidates for use in PAM as it is capable of providing strong PA signals. Furthermore, it can be designed in a wide range of sizes ranging from 5nm-400nm to mimic the size of the drug molecules being used. Thus, using AuNPs as a representative model drug for PAM imaging, researchers can extend the use of OR-PAM in the non-invasive characterization of the developed drug delivery platforms.

In OR-PAM imaging, visible light wavelength ($\sim$ 570nm) is used to enable imaging of blood vasculature to study if MN penetration resulted in any potential blood

vessel damage. If deeper imaging into skin tissue (up to a depth of 3.2mm) is required, near infrared (NIR) wavelength window can also be used with the developed PAM system. In this case, drugs of interest can be co-loaded with small molecule NIR dyes before MN-based delivery in tissues. A detailed study of the various types of drug carriers and nanoparticles compatible for use with OR-PAM or NIR-PAM would be beneficial to drive the potential use of this technology in MN development.

Thus far, in the development of novel polymer-based MN arrays substantial works have been achieved. Yet, more studies are necessary to achieve clinical translation of the technologies demonstrated. In the development of conductive MN array, it has been shown that using a dissolvable HA polymer results in a quick and bolus delivery of the encapsulated drug and PEDOT:PSS particles. While such a design has been shown to greatly reduce skin resistance and facilitate enhanced iontophoresis driven drug delivery into deeper tissues, certain drawbacks are identified. For clinical translation, release of polymer particles into skin is not widely favoured. As such, the design of the conductive MN array needs to be refined to allow minimal release of PEDOT:PSS particles.

A promising alternative would be to use hydrogel-forming polymers in the fabrication of conductive MNs as the pore size of hydrogel-forming polymers can be modulated using different degrees of crosslinking. It is further postulated that hydrogel forming conductive MNs can act as dry electrodes at the tissue interface upon application to further influence the drug delivery rate modulated by iontophoresis. Additionally, hydrogel-forming polymers also offer greater advantages when used in conjunction with iontophoresis. Literature has shown that hydrogel-forming MNs are superior in comparison to dissolving MNs in accelerating iontophoretic effects. This is

mainly because the application of an electrical current will lead to a dramatic increase in the swelling of the hydrogel-forming MN due to the enhanced water uptake by electroosmosis. This is capable of a 5-fold increase in the MN surface area that can greatly facilitate and enhance the movement of charged molecules[162]. Thus, a novel hydrogel-forming conductive MNs for achieving deeper drug penetration effect when used with iontophoresis can potentially bring about greater clinical significance.

Next, in the development of a MN-based glucose sensor promising *in vitro* work has shown the potential of quick colorimetric detection of glucose. Moving on, *in vivo* studies using diabetic mice models need to be done to validate the sensitivity and reliability of the developed glucose sensor in the determination of ISF glucose concentration. As *in vivo* conditions greatly vary from *in vitro* agarose studies, a detailed study to evaluate the optimum MN application duration and amount of ISF extracted will need to be carefully identified first. The reliability of results from the MN sensor will be confirmed via simultaneous detection of blood glucose values. In the current development, quantification of color change is determined using UV-VIS spectrophotometer and correlated to glucose concentration. To ease application use, colorimetric detection needs to be integrated with a proper detection tool such as a smartphone for easy read-out and interpretation of glucose concentrations and glycaemic conditions respectively.

APPENDIX

LIST OF PUBLICATIONS

JOURNAL ARTICLES

- 1) **Razina Z Seeni**[#], Xiaojun Yu[#], Hao Chang, Peng Chen, Chenjie Xu*. “Iron-oxide nanoparticles powered micro optical coherence tomography for in situ imaging the penetration and swelling of polymeric microneedles in skin.” ACS Applied Materials & Interfaces (2017)
- 2) Hao Chang, Mengjia Zheng, Xiaojun Yu, Aung Than, **Razina Z. Seeni**, Rongjie Kang, Jingqi Tian, Duong Phan Khanh, Linbo Liu, Peng Chen, Chenjie Xu*. “A Swellable Microneedle Patch to Rapidly Extract Skin Interstitial Fluid for Timely Metabolic Analysis.” Advanced Materials (2017)
- 3) Mohesh Moothanchery[#], **Razina Z. Seeni**[#], Chenjie Xu*, Manojit Pramanik*. “In vivo studies of transdermal nanoparticle delivery with microneedles using photoacoustic microscopy”. Biomedical Optics Express (2017)
- 4) **Razina Z. Seeni**, Chenjie Xu*. “Colorimetric glucose sensing based on rapidly swellable MN array.” (In preparation)
- 5) **Razina Z. Seeni**, Daniel Lio, Christian Wirajaa, Yuchun Liu, Bee Tin Goh, Chenjie Xu*. “Decreasing Skin Electric Resistance using PEDOT:PSS-Containing Conductive Microneedles in Transdermal Drug Delivery.” (In preparation)

BOOK CHAPTERS

- 1) **Razina Z. Seeni**, Sangeetha Krishnamurthy, Juliana M. Chan*. “Nanoparticles for Improved Topical Drug Delivery for Skin Diseases”.

Perspectives in Micro- and Nanotechnology for Biomedical Applications, pp.275-294. (2016) Imperial College Press.

- 2) **Razina Z.Seeni**, Lim Rebecca, Liu Yuchun and Xu Chenjie* “Iontophoresis in Transdermal Drug Delivery.” Imaging Technologies and Transdermal Delivery in Skin Disorders. (In preparation)

CONFERENCE SUBMISSIONS

- 1) Xiaojun Yu, **Razina Z.Seeni**, Linbo Liu, Chenjie Xu*, “ Polymeric Microneedles’ Swelling Property Characterization using Micro-optical Coherence Tomography,” Oral; Lasers and Electro-Optics Pacific Rim Conference, July 2017, Hongkong.
- 2) **Razina Z.Seeni**, Xiaojun Yu, Hao Chang, Peng Chen, Linbo Liu, Chenjie Xu*, “Iron Oxide Nanoparticle-Powered Micro-Optical Coherence Tomography (μ OCT) for Real Time in Situ Imaging Polymeric Microneedles Behaviours in Skin”; Poster; 2nd International Biophotonic Conference, November 2017, Singapore.
- 3) Mohesh Moothanchery, **Razina Z.Seeni**, Chenjie Xu, Manojit Pramanik*, “Photoacoustic Microscopy Imaging for Microneedle Drug Delivery”, Poster; Photons Plus Ultrasound: Imaging and Sensing Conference, February 2018, San Francisco.
- 4) **Razina Z.Seeni**, Lio Daniel, Yuchun Liu, Goh Bee Tin, Chenjie Xu*, “Conductive Microneedle-Mediated Iontophoresis For Non Invasive And

Painless Local Anaesthesia in Dentistry”, Poster; Microneedles 2018
Conference, May 2018, Vancouver.

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