Ultrasound and insertion force effects on microneedles based drug delivery: experiments and numerical simulation

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Ultrasound and insertion force effects on microneedles based drug delivery: experiments and numerical simulation

by

Han Tao

A doctoral thesis submitted in partial fulfilment of the requirements for the award of Doctor of Philosophy of Loughborough University

November 2015
This thesis is dedicated to my beloved parents - for their endless support and encouragement.
Abstract

Transdermal drug delivery (TDD) is limited by high resistance of the outer layer of the skin, namely *stratum corneum* which blocks any molecule that is larger than 500 Da. Research on TDD has become very active in recent years and various technologies have been developed to overcome the resistance of the *stratum corneum*. In particular, researchers have started to consider the possibility of combining the TDD technologies in order to achieve further increment for drug permeability. Microneedles (MNs) and sonophoresis are both promising technologies that can perform notable enhancement in drug permeation via different mechanisms and therefore give a good potential for combining with each other. We discuss the possible ways to achieve this combination as well as how this combination would increase the permeability. Some of the undeveloped (weaker) research areas of MNs and sonophoresis are also discussed in order to understand the true potential of combining the two technologies when they are developed further in the future. We propose several hypothetical combinations based on the possible mechanisms of MNs and sonophoresis.

Sonophoresis are widely used for increasing permeability of the skin for TDD. However, in the delivery of large molecules, sonophoresis alone cannot provide sufficient permeability enhancement. In addressing this issue, we propose optimised sonophoresis combined with solid MNs to further increase the permeation rates. Bovine serum albumin (BSA) (66 kDa), insulin (5808 Da) and lidocaine (234.34 Da) are used as model drugs of larger molecular weight to perform diffusion experiment in order to explore the enhancement of this combination for TDD. Franz diffusion cells were used to measure permeation through porcine skin after pre-treatment with a stainless steel microneedle array and 20 kHz sonophoresis for 10 minutes. The results confirmed the combination of MNs with sonophoresis can be considered as a stable method for delivery of large molecule weight drugs.

The permeation of large drug molecules affected by different insertion forces on MNs are also discussed. Many experiments conducted in the literature have investigated the effect of MNs on insulin permeation across skin. There are also a number of papers focused on the insertion force required for MNs successfully piercing into skin. However, there is little known on quantifying the relationship between the effect of MN insertion force and the amount of insulin permeated for given MNs. We have addressed this subject by using 1100 µm and 1400 µm long MNs to conduct in vitro permeability experiments on porcine skin with insulin as the target drug molecule. Histological images of MN treated skin are obtained from a microtome and the viscoelastic properties of the skin sample are measured using a
An in-house insertion force device is utilised that can reproducibly apply a certain force on MNs for a set period of time maintained by compressed air. The amount of MN force applied to porcine skin was shown to be related to the amount of target molecule permeated. An increase in insertion force increase the amount of insulin permeated. It was also demonstrated that using insufficient force may have reduced or prevented the amount of insulin passing through the skin, regardless of the geometry of the MNs.

The current status and challenges of the MNs related modelling and simulation are stated. Adequate modelling and simulation of MNs based drug delivery process has great potential to reduce the financial and time cost on the MNs studies and manufactures. A major limitation in this area is the lack of information on the structural and mechanical properties of skin. Conventionally, the mathematical models of MNs drug delivery define the shape of the holes created by the MNs in the skin as the same as their actual geometry. The skin deformation caused by the MNs must be deduced from the viscoelastic properties of the skin. However, the histological images of the MN treated skin indicate that the real insertion depth is much shorter than the length of the MNs and the shapes may vary significantly from one case to another. This will make accurate prediction on the skin deformation extremely difficult because the properties of the skin is complicated and the insertion condition are also affecting the results. In addressing these points, we propose a new approach for modelling MN based drug delivery, which incorporate the histology of MN pierced skin using a number of concepts borrowed from image processing tools. It is expected that the developed approach will provide better accuracy of the drug diffusion profile. A new computer program is developed to automatically obtain the outline of the MNs treated holes and import these images into computer software for simulation of drug diffusion from MN systems. This method can provide a simple and fast way to test the quality of MNs design and modelling, as well as simulate experimental studies, e.g., permeation experiments on MN pierced skin using diffusion cell. The numerical simulation results of permeation using insulin and lidocaine are compared with experimental results under the same conditions. They show strong correlations and confirm that the new model can simulate drug diffusion with good accuracy.

Keywords: transdermal drug delivery, microneedles, sonophoresis, Franz diffusion cell, permeability, numerical modelling and simulation, insertion force.
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List of Publications

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**Oral presentation**

Enhancement for Transdermal Delivery of Large Molecule Using Low Frequency Sonophoresis Combined with Microneedles

Presenters: Tao Han and Diganta Das, Loughborough University, UK

Venue: The third International Conference on Microneedles, University of Maryland School of Pharmacy, USA.

Date: May 19, 2014.

**Poster presentation**

Permeability enhancement for transdermal delivery of large molecule using low frequency sonophoresis combined with microneedles, the third international conference on microneedles, Maryland, USA.

Ultrasound Enhanced Drug Delivery based on Microneedles, annual research conference of Loughborough University, Loughborough, UK.

Permeability enhancement for transdermal delivery of large molecule using low frequency sonophoresis combined with microneedles, the 6th APS international PharmSci 2015, Nottingham, UK.

A new paradigm for numerical simulation of microneedle based drug delivery aided by histology of microneedle pierced skin, the 6th APS international PharmSci 2015, Nottingham, UK.
Chapter 1
Introduction

1.1 Background

Transdermal drug delivery (TDD) is a widely used drug delivery technique for many therapeutic applications and it is considered as an alternative to oral, intravascular, subcutaneous routes etc. (Solanki et al., 2012). The drug delivery mechanism of TDD is governed by passive diffusion which can be further divided into two transport trajectories: the lateral transport along the phospholipid bilayer (lateral diffusion) (Almeida et al., 1992), and the transport across the bilayer (transbilayer transport) (Lieb and Stein, 1986). The lateral diffusion through skin is generally achieved in three pathways: sweat glands, hair follicles (sebaceous glands) and intercellular spaces. This implies that the passive diffusion rate is restricted because of the smaller drug transport area of these pathways. The fractional areas of sweat glands, hair follicles and intercellular spaces over the whole skin area are 1/1000, 1/10000 and 1/500, respectively (Walters and Roberts, 2002). On the other hand, the transbilayer transport can be applied on most of the skin area but it is limited by the skin property.

A drug that is administrated in these ways must be fixed in a certain range of molecular weight (< 500 Da) (Brown et al., 2006) and partition coefficient (K<sub>ow</sub>) ranging between 1 - 5 (Kushner, 2006; Kalia and Guy, 2001).

The stratum cornuem (SC) which is the outermost layer of skin blocks any drug molecule that is not in the aforementioned range. Researches on large molecules delivery become more active and various technologies have been applied conquer the problem. Chemical enhancers are used to tentatively alter the skin structure or modify the drug/skin partition coefficient to reduce the resistance of SC layer (Asbill et al., 2000). Gene gun (Zhang et al., 2013) intends to deliver the drug molecules by directly shooting it into skin with the help of particle accelerator. Ultrasound is employed for cavitation effects on the SC layer over time, which induces pressure field to increase drug delivery rate with convection flow (Herwadkar et al., 2012). The convection flow can be also motivated by electric field which is known as iontophoresis (Banga et al., 1999). Besides the above methods, MNs can disrupt the skin where new pathways are created for the drug molecules to pass through (Nayak et al., 2013). However, these technologies do not necessarily provide satisfactory permeability of drugs;
thereby, the problem of TDD may still unsolved. Therefore, development and optimization on current techniques are vital for the permeation increment of large molecules.

The MNs are the most efficient and promising technique among other TDD methods. However, the size and alignment of the MNs patch are limited to maintain the administration process pain-free. To understand how to maximize the effect of MNs enhanced drug delivery, numerical simulation should be done which is an efficient way to examine the feasibility of the designed MNs. Conventionally, the MNs model for simulation is based on the geometry of the MNs where the computational domain is reproduced from the exact shape of the designed MNs (Aggarwal and Johnson, 2004). The deformation of the skin is then deduced from the specific viscoelastic properties of the skin sample. Due to the complicated structure of the skin, the parameters that describe the viscoelastic properties of skin are difficult to be measured or cannot fully represent the material’s nature. Experimental factors such as the insertion forces, insertion durations, and the integrity of the SC layer after insertion are causing even more decrement on the accuracy of simulation. A reliable modelling method is thereby demanded for the purpose of acquiring high quality simulation results.

1.2 Aims and objectives

The overall aim of this research is to explore a methodology involving ultrasound that can increase the permeability of drug molecules through skin and develop advanced model to predict the diffusion profiles. The specific aims of the PhD study are as follows:

- To study current techniques in TDD to understand all the advantages and limitations based on their mechanisms.
- To identify and explore methods that can increase the permeability of large molecules in TDD.
- To carry out an experimental study on the selected method to discover the efficiency of this method for the delivery of different sizes drug molecules.
- To optimize the parameters for the selected enhancement method to maximize the diffusion rate of drug molecules.
- Reveal the relationship between the input parameters for the experiment and the diffusion profile of the drug molecules.
- Study current MNs modelling methods and try to develop an advanced modelling paradigm that can provide reliable simulation results.
• Employ the new modelling method to simulate different types of MNs as well as assorted experimental conditions.

1.3 Thesis structure

A brief description of each chapter of this thesis is presented as follows:

Chapter 2: This chapter is a review of the literature to introduce context that related to the research. It is consisted of two sections: the first section has reviewed current technologies employed for the enhancement of permeability in transdermal drug delivery, particularly two main technologies which are MNs and ultrasound. The mechanisms of those two technologies have been introduced to elaborate the possible forms of combination based on those mechanisms. The second section is focused on presenting MNs related modelling and simulation. The subject is discussed in three different aspects: The first part provides a brief review of different types, materials and design of MNs. The second part discusses current parameters and tools involved for MNs modelling. The last part introduces the theoretical base for the optimization and some simulation results.

Chapter 3: This chapter is an experimental exploration on ultrasound enhanced transdermal drug delivery based on MNs. The solid MNs is employed to pierce the porcine skin in order to expose part of the viable epidermis, thereby increasing the permeability for drug molecules. The low frequency sonophoresis is then applied on the MNs treated area for further enhancement. The theoretical base of this combination is to use MNs creating tiny lesions on the porcine has already been discussed in the Chapter 2 so the outcome from the experiment confirms the feasibility of this method. Bovine serum albumin and lidocaine NaCMC/gel hydrogel are the two sample drugs used for diffusion experiment. The permeation results are including: passive diffusion, diffusion enhanced by MNs only, diffusion enhanced by ultrasound only, and diffusion enhanced by ultrasound combined with MNs.

Chapter 4: The results of the relationship between the insertion forces and drug permeability are presented in this chapter. Two large protein, bovine serum albumin and insulin, are chosen as sample molecules for the diffusion experiment. An in-house air pump, which mimics an applicator, is used to produce and control the insertion force. The insertion force varies from 17.3N to 69.1N and the diffusion profile under each insertion force is measured. The histological images of the cross-sectional view of the MNs treated skin is acquired using microtome to examine the real depth of the hole created by different insertion forces. There
is also a section comparing the diffusion result under different insertion forces with the diffusion results using ultrasound combined with MNs method.

Chapter 5: This chapter introduces a methodology for numerical simulation of MNs. The histological image which shows the cross-sectional view of the MNs hole is processed by a MATLAB program for the sake of acquiring highly accurate computational domain. The obtained domain can directly depict the deformation of the skin as well as the real insertion depth of the MNs, thereby considered as an advanced method comparing to the models only based on the MNs geometry. This method is then examined by different input models which are including: an ideal geometry of the hole obtained from drawing, histological images from microtome slice and optical coherence tomography. After the validity of this method has been verified, it is then extended to be used for 3D modelling. There are also simulation results that are deduced from histological images with different insertion forces which are then compared with the results from chapter 4.

Chapter 6: This chapter summarizes the experimental and theoretical results from the thesis. Furthermore, some possible future work in continuation of this work is discussed.
Chapter 2
Literature review

Overview

This review chapter is consisted of two subchapters: The first section discusses the potential of combined ultrasound and MNs for enhanced transdermal drug permeation and the second section discusses MNs related modelling and simulation. The first subchapter is dedicated to deliver the ideas of permeation enhancement in transdermal drug delivery (TDD) which is limited by the outer layer of the skin, i.e., the stratum corneum. Research on TDD has become very active in recent years and various technologies have been developed to overcome the resistance of the stratum corneum to molecular diffusion. In particular, researchers have started to consider the possibility of combining the TDD technologies in order to have further increase in drug permeability. MNs and ultrasound are both promising technologies. They achieve enhancement in drug permeation via different mechanisms and therefore give a good potential for combining with each other. This review will focus on discussing the potential of this combinational technique along with other important issues, e.g., the mechanisms of ultrasound and MNs as it is these mechanisms which are coupled via the two systems (i.e. MNs and ultrasound). We discuss the possible ways to achieve this combination as well as how this combination would increase the permeability. Some of the undeveloped (weaker) research areas of MNs and sonophoresis are also discussed in order to understand the true potential of combining the two technologies when they are developed further in the future. We propose several hypothetical combinations based on the possible mechanisms involved in MNs and ultrasound. Furthermore, we carry out a cluster analysis by which we determine the significance of this combinational method in comparison with some other selected combinational methods for TDD (e.g., MNs and iontophoresis). Using a time series analysis tool (ARIMA model), the current trend and the future development of combined MNs and ultrasound are also analysed. Overall, this review section indicates that combining MNs and ultrasound is a promising TDD method for the future.

The second section discusses the current status and challenges of the MNs related modelling and simulation. Adequate modelling and simulation of MNs based drug delivery process has great potential to reduce the financial and time cost on the MNs studies and manufactures. This chapter introduced the current states of the MNs modelling research including the design, modelling, simulation and optimization of the MNs. The theoretical
basis for simulation of MNs enhanced diffusion is also presented in details. Developing models and simulation techniques to adequately predict performance of MNs is key to achieving microneedle systems with desired performance.

2.1 Potential of combined ultrasound and microneedles for enhanced transdermal drug permeation

2.1.1 Background

Transdermal drug delivery (TDD) methods intend to deliver drug molecules to the blood circulation at a controlled rate for which the molecules need to pass through different sub-layers of the skin. TDD is developing fast and there are now many approved drugs for TDD, e.g., nineteen (19) drugs have been approved by the Food and Drug Administration, USA (Ita, 2014). The potential of TDD for treating human diseases is also huge. For example, TDD can provide prolonged treatment time in the cure of chronic diseases while maintaining the permeation of the active drug molecules at a controlled level (Jampilek, 2013). The diseases may be either psychological or physiological, and may need TDD ranging from nicotine patch for smoking cessation to the treatment of eczema (Christopher and David, 1994; Darsow et al., 1996). However, the full potential of TDD is not fully exploited yet, which is evidenced by the fact that new questions continue to be asked on how to develop the TDD methods further, for example, to resolve specific issues and/or incorporate the latest technological advances. For instance, it has been asked if it is possible to make functionalised delivery system for vaccines that can be applied in a simple way such as topical administration (Barry, 2001). To develop a TDD method for clinical purposes, one may require a significant amount of finances and many technical impediments would need to be resolved (Banga et al., 2013). For example, it is evident that the market of TDD products has developed very fast and they were worth a market value of US $21.5 billion in 2010 which accounts for more than 12% of global drug delivery market. The development of the TDD market is predicted to reach US $31.5 billion by 2015 in which US $3 billion belongs to transdermal patch market (PharmaLive Special Report, 2015). However, the diversity of the drugs that could be delivered and various applications of these TDD techniques for treating human diseases are still limited.

Despite the commercial successes of the TDD methods, further development and success of these methods cannot solely depend on the transdermal patches. Improvement on the drug delivery efficiency and increment on the numbers of applicable drug molecules need to be
achieved in the future by extending the TDD technology in multiple ways. In these regards, one of the main technical obstacles that should be overcome is the low efficiency on delivering large molecules such as proteins, vaccines, and micro-particles (Van Der Maaden et al., 2012) using the TDD methods. MNs (Patil et al., 2012) and ultrasound (Merino et al., 2003) are two TDD techniques which work using different principles/mechanisms but they have shown great potential to remove this obstacle either on their own or in combination with each other. There are a number of publications now which have reviewed these two technologies on their own (Pierre and Rossetti, 2014; Sunil et al., 2013; Schoellhammer et al., 2014; Quinn et al., 2014; Azagury et al., 2014). There are also some recent studies where MN and ultrasound have been combined to increase skin permeability of large molecule (Han and Das, 2013; Nayak et al., 2014). However, there is a lack of systematic review which discusses thoroughly the potential of combining MNs with ultrasound for enhanced drug permeation. Therefore, this review will focus on discussing the possible ways by which these two technologies could be combined. The first section of this review will focus on explaining why the combination of MNs and ultrasound is important for TDD. The second and third sections will review the mechanisms of ultrasound and MNs, respectively, as these are the keys in the success of a TDD method that combines MNs and ultrasound. The fourth section will discuss the possible ways of combination and try to suggest what combinations one may be interested in the future with the help of a cluster analysis. The last section is the summary section of the review. The scope of this review is discussed further in detail later in this section.

### 2.1.1.1 Roles of TDD

In order to provide further context to this review, we discuss the roles of TDD method briefly in this section. Not until the 1940s, has the TDD been specialized as one of the most essential drug delivery methods including the parenteral delivery (hypodermic injections) and oral formulations (solutions, suspensions, tablets and capsules) (Hillery et al., 2001). The main advantages of TDD over drug delivery through other routes are that: (a) TDD is user friendly, so that it can prevent needle phobia and avoid the pain perceived during the parenteral delivery (Subramony, 2013), (b) TDD can dodge the gastrointestinal and liver metabolisms which are the most common issues in oral drug delivery (Hadgraft and Guy, 1989) and, (c) TDD can provide long-term treatment without causing significant inconvenience, e.g., patients do not need to carry bulky medical instruments during the intravenous therapy which usually takes many hours (Yang et al., 2004). In the past, the TDD methods mostly involved the uses of skin ointments and creams until a great progress
was made in the 1980s when a transdermal patch was first introduced for the treatment of space motion sickness aimed at delivering scopolamine by attaching the transdermal patch on the back of the ear (Ashton, 1981). In general, the transdermal patches can prevent evaporation during treatment as well as achieve control rates of drug delivery (Davarana et al., 2005). However, their mechanism for drug delivery is based on passive diffusion. For this reason, the outermost layer of the skin, i.e., the stratum corneum (SC), restricts the choice of the drug molecule that can be administrated. For example, the molecular weight (MW) cut off for these molecules is generally taken to be under 500 Da (Brown et al., 2006) while their partition coefficient ($K_{ow}$) should be between 1 to 5 (Kalia and Guy, 2001; Kushner, 2006).

2.1.1.2 Different TDD technologies

It is a matter of fact that the transdermal patch is a low efficient method in terms of drug permeability and area of skin covered by drug transport. However, there are a number of other technologies which particularly aim to increase drug transport rate and they may extend the diversity of the drug molecules that may be used in TDD (e.g., MNS (Lee et al., 2008), sonophoresis (Paliwal et al., 2006) and iontophoresis (Kalia et al., 2004)). All of these technologies are non-invasive or minimally invasive and, thus they provide painless drug administrations.

The technologies that aim to enhance the permeability of the drugs through the skin as compared to transdermal patches alone can be grouped broadly according to the following five classifications:

- Methods that adjust the physicochemical properties of the drug molecules or increase the chemical potential of the drug solution to acquire better delivery rate, e.g., prodrug (Barry, 2001);
- Methods that tentatively alter the skin structure or modify the drug/skin partition coefficient to reduce the resistance of stratum corneum, e.g., chemical enhancers (Asbill et al., 2000; Johnson et al., 1997);
- Methods that deliver drugs or microparticles directly into skin with the help of particle accelerator, e.g., gene guns (Zhang et al., 2013; Zhang et al., 2014; Zhang et al., 2013; Zhang et al., 2013);
- Methods that use a gradient field (e.g., pressure gradient, electrical charge, any others) to induce convective flow increasing drug delivery rate, e.g., iontophoresis (Banga et al., 1999) and sonophoresis (Herwadkar et al., 2012);
• Methods that physically disrupt or damage the skin to create new pathways which allow the drug molecules to be delivered through the skin barrier, e.g., MNs (Nayak et al., 2013).

These five approaches are shown in more detail in Figure 2.1. From the figure we can see that some TDD techniques may increase the diffusion rate via multiple mechanisms (sonophoresis, electroporation, etc.) while others may work similarly under the same categories. The combination of more than two or more than two techniques under the same category may not be able to yield a promising permeability increment due to the possible redundancy/suppression of a particular mechanism in presence of another, e.g., MNs and SC removal methods are both under category (iv) that aiming to bypass SC layer physically, thereby combining these two methods will be unnecessary. On the contrary, some TDD approaches indicate more potential for combinational methods because there are improved possibilities for them working in a synergetic way with another approach. Because the categories in Figure 2.1 are subjectively divided, they are not necessarily able to provide every possible patterns of combination.

Figure 2.1 Current TDD technologies can be presented in five broad branches
As stated earlier, the existing work in the literature suggests that it is possible to combine more than one method for enhancing drug permeability and there is a significant amount of work on different combinational approaches (Sachdeva et al., 2013; Shetty et al., 2013). However, it needs to be pointed out that the researches on the development of individual technology are very important for the development of the combinational methods because the researches on the individual method can provide better understandings and stronger bases for the applications of these technologies. These improvements are crucial factors to ensure diversity and quality of the combinations.

The ultrasound and MNs combination has covered four branches (categories ii, iii, iv and v) in the Figure 2.1 which suggests that there could be much more possible forms of combination in the future. To have better understanding on the possible combinations between ultrasound and MNs, a detailed review based on the mechanisms of both technologies are necessary which has been carried out in following sections. In order to discover more opportunities in the ultrasound and MNs combination, the main mechanisms of these two techniques will be reviewed individually. However, there are also many other minor factors among those mechanisms which could be important in some circumstances or will become significant factors when accuracy of the TDD is taken into account. Therefore, these factors and their main mechanisms will be discussed (section 2.1.4).

2.1.2 Ultrasound applications in TDD

The ultrasound participated applications cover many cross-cutting research areas which include physics, chemistry, biology, engineering, and others. One of the main areas where ultrasound has been employed is the medicine, as exemplified by a large number of publications between 1975 and 2013 (database searched: Scopus; keyword: ultrasound). During this period, approximately 210,540 publications have appeared which relate to the use of ultrasound in medicine alone whilst the total numbers of papers relating to all areas of ultrasound applications are approximately 283,430. The number of papers of ultrasound applications for medicine only was 941 in 1975 and it reached 13,470 in 2013 which suggests an average 7.25% increment in the number of publications each year. The publications of medical ultrasound divided by total number of ultrasound publications are shown in Figure 2.2 as percentage to present the weight of medical ultrasound research from 1975 to 2013.

Generally, the ultrasound applications in medicine depend on the power and frequency of the ultrasound output (Haar, 2007). The intensity of the ultrasound is a crucial parameter
which determines its usage for either diagnostic or therapeutic purpose. The diagnostic ultrasound must have a relatively low intensity to reduce any adverse effect to human body whilst high intensity ultrasound can damage tissues via cavitation and high temperature. The ultrasound applications classified according to their frequencies and intensities shown in Table 2.1.

Figure 2.2 The numbers of publications of medical ultrasound over all ultrasound publications (all results searched using Scopus at 2013) (Scopus, 1995).

Table 2.1 The applications of ultrasound sorted by different parameters

<table>
<thead>
<tr>
<th>Diagnostic ultrasound</th>
<th>Physiotherapeutic ultrasound</th>
<th>Sonophoresis</th>
<th>High intensity focused ultrasound</th>
</tr>
</thead>
<tbody>
<tr>
<td>(real time medical imaging (Sarvazyan AP, Urban MW, Greenleaf JF, 2013))</td>
<td>(bone healing (Dyson M, Brookes M, 1983))</td>
<td>(transdermal drug delivery (Smith NB, Lee S, Shung KK, 2003))</td>
<td>(ultrasound blade (Haar GT, 1995))</td>
</tr>
</tbody>
</table>

Intensity: Low ➔ High

<table>
<thead>
<tr>
<th>1-18MHz</th>
<th>1-3MHz</th>
<th>20kHz-3.5MHz</th>
<th>1-5MHz</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-cavitation</td>
<td>Cavitation involved</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
According to Table 2.1, sonophoresis (or phonophoresis) can be defined as an ultrasound application which has sufficient intensity to reduce the resistance of skin but keeps the temperature within a safe range. The first reported application of sonophoresis was used to treat polyarthritis by using hydrocortisone ointment combined with ultrasound in 1950 (Fellinger and Schmidt, 1954). Since then this method has been widely used in the treatment of many other diseases including bone joint diseases and bursitis (Newman et al., 1958). Although this approach is recognized by scientists, a number of issues (e.g., how to choose the parameters of ultrasound) continue to pose problems in sonophoresis.

Besides the intensity and frequency of ultrasound, there are other parameters such as the duty cycles of the ultrasound, the treatment time and, the distance between ultrasound transducer and target, which also need to be considered for specific ultrasonic application (Mitragotri and Kost, 2004). Although they are often treated as minor factors (Nyborg et al., 2002), their importance cannot be underestimated and could become more useful in the future.

### 2.1.2.1 Impediments in the development of sonophoresis based TDD methods

There a number of inherent mechanisms in the sonophoresis based TDD based methods which affect their performances. These methods have been involved in a lot of branches in TDD research as discussed earlier. However, it seems that the sonophoresis research is still not very active if compared with other TDD methods. To illustrate this point clearly, we have carried out a time series analysis based on an autoregressive integrated moving average (ARIMA) model. We apply this analysis to illustrate the development of sonophoresis research over time. ARIMA is a common approach used for time series analysis of quantitative data and it can be used to determine trends of the data by evaluating the projection from past patterns. This approach can be particularly useful to model trends where the data have non-linear and fluctuating trends (such as the data in this work). ARIMA has been widely applied in social science areas to help people for making informed decisions. For example, the approach can help one (i) to find opportunities by analysing the market trends (Hosseini, 2013), (ii) to set right deposit rates in a bank by studying the money transaction data (Cheshti et al., 2014), (iii) to predict the traffic flow to help people to avoid traffic congestion (Wang et al., 2014). At the moment, there is no such time series analysis
for the trend of sonophoresis or MNs research. ARIMA uses univariate Box-Jenkins models which imply that only the past values of the variables are involved in the analysis and it does not consider data from other series (Pankratz, 1983). The model consists of two parts: autoregressive (AR) and moving average (MA) which can be chosen together or individually depending on the modelled situation. The model applied for the purpose of this review is MA (1) which means no AR method is employed. The number in the brackets indicates an order of the algorithm.

For the time series analysis, the data acquired from scientific paper database are differentiated twice with respect to time to achieve a stable model, which provides a time series constant for the data. In the present case, the estimated time series constant was 2.029. MA (1) model is then employed to fit the trend of sonophoresis publications per year (Figure 2.3). The fit and forecast results using the MA (1) model are shown in Figure 2.3. The predicted results on the number of publications in year 2020 are 60 which suggest a slight increment of 1.08% per year from the year 1970. Although the ultrasound technology is well understood, the results of this analysis shows that the development of sonophoresis is relatively slow and, there is no sudden increase or decrease in its interests as it may happen in many other methods or techniques. The number of publications on sonophoresis research has an averaged increment of 9.05% per year from 1970 to 2013. We believe there are two main reasons that have led to this slow growth as discussed below.

Firstly, the sonophoresis based TDD methods are not supported by well-developed theory. For instance, the current theoretical description of the ultrasound assisted molecular diffusion and convection is simply an extension of the hindered diffusion theory as shown in Equation (2.1) (Tang et al., 2000):

\[
\log P_{\text{diff+conv}}^{US} = \log \frac{\text{Pe}}{1 - \exp(-\text{Pe})} + \log \left( \frac{KT}{2z^2F_c e_0 D_{\text{ion}} H(\lambda_{\text{ion}})} \right) + \log R
\]

Where \( P \) is the drug permeability [m/s], \( \text{Pe} \) is the Peclet number [-], \( k \) is Boltzmann constant [J/K], \( T \) is the absolute temperature [K], \( z \) is the electrolyte valence [-], \( F \) is the Faraday constant [C/mol], \( c \) is the electrolyte molar concentration [mol/m3], \( e \) is the electronic charge [C], \( D \) is the diffusion coefficient [m²/s], \( H(\lambda) \) is the hindrance factor [-] and \( R \) is the skin resistivity [Ω·m]. The size and electrical properties of the molecules have been considered in this model, but there is little or no theoretical development work on the effects of the
modification to the skin due to cavitation effect which is the main factor that sonophoresis contributes to TDD. Well-developed theoretical descriptions of various mechanisms in sonophoresis based TDD are important not only for its further development and research but also for understanding the experimental data and combining ultrasound to other TDD methods.

Secondly, it seems that the researches on sonophoresis experiments involving drug permeability are somewhat chaotic and lack consistency. For example, the skin samples used in the experiments may lack a consistent quality and standard. Factors such as imperfections in skin samples, skin thickness variations, different sampling areas of the skin, different skin types, etc. also affect the drug permeability especially in the transport of large molecules. Furthermore, the uncertainties in the ultrasound system may alter the diffusion results. These uncertainties are included in the performance of the ultrasound system which needs to be acquired using a hydrophone or force balance, and other details of the ultrasound setup (e.g., parameters such as distance of the ultrasound horn to skin surface and localized transport regions measurement (Polat et al., 2012)). Additionally, the significance of different mechanisms is not well understood and they must be determined. As mentioned earlier, sonophoresis includes several different mechanisms, all of which contribute to the molecular diffusion. The same ultrasound output, drug molecules and skin samples under different experimental conditions may show different diffusion results for

Figure 2.3 Fit and forecast results of the trend of sonophoresis publications using MA (1) model developed in MATLAB (keywords for search: sonophoresis/phonophoresis using Scopus (Scopus, 1995)).
ignoring some minor factor (Park et al., 2012; Escobar-Chávez et al., 2009). Thermal effect, acoustic streaming and other phenomena can be significantly magnified due to those unnoticed factors varying the diffusion results. However, these factors are impossible to quantify separately in practice. Therefore, the overall progress of the research which particularly employs experimental methods seems to be slow. An illustration of percutaneous delivery of common drug molecules (MW are arranged from low to high) enhanced by sonophoresis is shown in Table 2.2. The table presents different diffusion results of a number of drug molecules. Even though all the experimental results have shown permeability increases, the data need to be carefully used in the context of sonophoresis. Therefore, the researchers have to ignore the less significant mechanisms in order to combine the sonophoresis to other TDD methods. To extend the combination range of sonophoresis and to increase the quality of combinations, sonophoresis experiments must be regulated and more elaborate experiments in consideration of the minor mechanisms must be executed.
Table 2.2 Ultrasound enhanced diffusion experiment data of selected compounds (mannitol, sucrose, cortisol, calcein, inulin, and insulin).

<table>
<thead>
<tr>
<th>Solute property</th>
<th>Main experimental apparatus used</th>
<th>Skin type</th>
<th>Freq uency (W/cm²)</th>
<th>Intensity (W/cm²)</th>
<th>Exposure condition</th>
<th>Composition of donor solution in experimental apparatus</th>
<th>Receiving solution in experimental apparatus</th>
<th>Analytical apparatus</th>
<th>Temp. (°C)</th>
<th>Data analysis</th>
<th>Result summary</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mannitol</td>
<td><em>In vitro</em> US transducer; Franz diffusion cell</td>
<td>In vitro</td>
<td>1.1</td>
<td>0.1</td>
<td>CW</td>
<td>5% v/v [14C] mannitol dissolved in 100ml ethanolic solution</td>
<td>Liquid scintillation counter, 24-33ml distilled water, 1.1ml aliquots were withdrawn every 30min within 5h</td>
<td>Receptor solution: 37</td>
<td>Mean ± S.D. over 5h</td>
<td>Mean flux of radiolabeled [14C] mannitol under US treatment of 0/0.1/2 W/cm² are: 90.51±19.51/not detectable/375.7±53.21 pmol/cm²/h respectively; Not detectable because mannitol is mainly mediated <em>via</em> transfollicular pathway, but under 0.1 W/cm² US the sebaceous sebum is released into hair follicle shaft. For histological results, see Fig 3 in the original paper (Meidan et al., 1998).</td>
<td></td>
</tr>
<tr>
<td>MW: 182 Da</td>
<td><em>In vivo</em> on the upper back of hairless SD rats aged 4-5 months</td>
<td>Freshly excised surgical US transducer</td>
<td>1.0</td>
<td>1.5</td>
<td>CW</td>
<td>1µCi/µl D-[3H]mannitol saturated with 20µl 90% ethanol and 10% water</td>
<td>Liquid scintillation counter, Urine in bladder collected using catheterization every 15-30 min within 2h</td>
<td>Room temperature</td>
<td>Mean ± S.E.M. and student’s t test, P&lt;0.05 over 5h</td>
<td>Mean secretion rate of radiolabeled [3H] mannitol shows 20-fold higher in the US treated group (n=4) than in the controls (n=12); for detailed data see Fig. 1 in the original paper (Levy et al., 1989).</td>
<td></td>
</tr>
<tr>
<td>Log Kow = -3.10</td>
<td><em>In vitro</em> on the upper back of hairless SD rats aged 4-5 months</td>
<td>Modified Franz diffusion cell; US transducer</td>
<td>1.1</td>
<td>1.5</td>
<td>CW</td>
<td>5µCi/ml [3H]mannitol saturated in 3ml 0.9% normal saline, 500µl samples retrieved</td>
<td>Liquid scintillation counter, Receptor solution: 29-31, calculated over 24h</td>
<td>Room temperature</td>
<td>Mean ± S.E.M. calculated over</td>
<td>Mean flux with and without US are 0.4±0.15 (n=4) and 0.5±0.15 (n=4) pg/cm²/h, respectively. For histological results see Fig. 1 and 2 in the original paper.</td>
<td></td>
</tr>
</tbody>
</table>
Ultrasound and insertion force effects on microneedles based drug delivery: experiments and numerical simulation

(90% efficiency)

<table>
<thead>
<tr>
<th>In vitro</th>
<th>hairless</th>
<th>mice aged</th>
<th>6-7 weeks</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Sucrose</th>
<th>Franz diffusion cell, Epidermis</th>
<th>0.02</th>
<th>0.125</th>
<th>DC:10</th>
<th>1μCi/ml</th>
<th>15.8ml 0.01mol/l</th>
<th>Thermocouple, microsopy</th>
<th>Room temperature, circulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>MW: 342 Da</td>
<td>Log K_{in} = -3.70</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Log K_{in} = -3.70</td>
<td>hydrophone, human</td>
<td>heat</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>nylon mesh</td>
<td>skin</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Epidermis</td>
<td>heat</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>by a</td>
<td>from</td>
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<td>-</td>
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<td>-</td>
<td>-</td>
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<td>-</td>
</tr>
<tr>
<td>sonicator test</td>
<td>stripped</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>human</td>
<td>pre-treatment</td>
<td>0.01mol/l, NaCl 0.137 mol/l</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ethanolic solution</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1.1</td>
<td>0.1</td>
<td>CW</td>
<td>5% v/v</td>
<td>24-33ml distilled water, 1.1ml aliquots were withdrawn every 30min within 5h</td>
<td>Liquid scintillation solution: 37</td>
<td>Receptor over 5h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>US transducer;</td>
<td>hairless</td>
<td>male</td>
<td>Wistar rats skin</td>
<td>250-300g, aged 4-5 months</td>
<td>2.0</td>
<td>CW (5 min pre-treatment)</td>
<td>100ml ethanolic solution</td>
<td>-</td>
</tr>
</tbody>
</table>

Mean flux with and without US are 44.0±10.6 (n=5) and 27.1±5.5 (n=9) pg/cm²/h, respectively.

For histological results see Fig. 1 and 2 in the original paper (Macheta et al., 1998)

Mean flux of radiolabeled [14C] sucrose under US treatment of 0/0.1/2 W/cm² are 11.49±3.01/not detectable/51.36±2.62 pmol/cm²/h, respectively.

For histological results see Fig. 3 in the original paper (Meidan et al., 1998)
### Cortisol

**MW:** 362 Da  
**Log $K_{ow}$:** 1.61

**US transducer:** combined with iontophoresis devices (5mA pre-treatment for 20 min)

**In vivo patients with unilateral carpal tunnel syndrome**

<table>
<thead>
<tr>
<th>Group</th>
<th>Early Stage</th>
<th>VAS before/after treatment: 7.4±0.5/1.8±1.9;</th>
<th>Moderate Stage</th>
<th>VAS before/after treatment: 8.1±1.1/1.8±1.5;</th>
<th>Advanced Disease Stage</th>
<th>VAS before/after treatment: 8.0±1.2/4.2±1.9 (Dakowicz and Latosiewicz, 2005)</th>
</tr>
</thead>
</table>

- **A 23-gauge butterfly catheter into a cubital fossa vein; US transducer**
  - **In vivo human, aged 18-33 (X̄=25, SD=2.74)**

<table>
<thead>
<tr>
<th>1.0</th>
<th>1.0</th>
<th>CW (5 min)</th>
<th>30 ml aquasonic gel for control group; 30ml 10% hydrocortisone gel for experimental group</th>
<th>5 cc blood sample was drawn followed by 2 cc saline flush in different time point</th>
<th>Centrifuge; cortisol assay (0.45 µg/l sensitivity) &amp; blood sample clotted at room temp.</th>
<th>Two-way ANOVA, P&lt;0.05 over 30 min time period</th>
<th>The serum cortisol levels between US alone and hydrocortisone phonophoresis are:</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>10 min before US treatment: 9.7±4.1&amp;10.1±2.9 µg/dl;</td>
<td>2. Immediate after US treatment: 8.2±4.2&amp;8.8±3.5 µg/dl;</td>
<td>3. 5 min after US treatment: 8.2±3.8&amp;9.4±3.5 µg/dl;</td>
<td>4. 15min after US treatment: 7.6±3.6&amp;8.0±.3 µg/dl.</td>
<td>Four subjects reported intolerable heating (Bare et al., 1996)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- **Modified Franz Freshly excised**
  - **CW 6µCi/ml [3H]hydrocorti solution, 20% scintillation Receptor solution: 29- Mean: S.E.M. calculated over Mean flux with and without US are 3.5±1.3 (n=5) and 3.0±0.6 (n=5) pg/cm²/h, respectively. For**
**Ultrasound and insertion force effects on microneedles based drug delivery: experiments and numerical simulation**

<table>
<thead>
<tr>
<th>Diffusion Cell;</th>
<th>Surgical</th>
<th>Human Skin (90% Efficiency)</th>
</tr>
</thead>
<tbody>
<tr>
<td>In vitro</td>
<td>Hairless mice aged 6-7 weeks</td>
<td>Modified Franz diffusion cell; US transducer (10 min recalibration/h);</td>
</tr>
<tr>
<td>In vitro</td>
<td>Hairless mice aged 6-7 weeks</td>
<td>Intact Wistar rats skin 250-300g, aged 4-5 months, frozen in -20°C up to 1 month</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CW or DC: 33.3 (Length 2ms interval as above over 4h period)</td>
</tr>
</tbody>
</table>

- Sone saturated in 3 ml 0.9% normal saline
- PEG 400 and 20% ethanol, 500µl samples retrieved regularly with equal compensation
- Counter; light and electron microscopy
- Jacket: 32
- Mean flux over 24h with and without US are 46.8±4.6 (n=5) and 40.4±7.2 (n=5) pg/cm²/h respectively. For histological results see Fig. 1 and 2 in the original paper (Macheta et al., 1998)

| Mean flux: control/1.1MHz/3.3MHz are 0.0073±0.0105/0.0133±0.0016/0.0160±0.0052 pmol/cm²/h respectively; Oleic acid/Oleic acid+1.1MHz are 0.0494±0.0092/0.0583±0.0029 pmol/cm²/h respectively; Azone/Azone+1.1MHz/Azone+3.3MHz are 0.0407±0.0054/0.1021±0.0125/0.0953±0.0172 pmol/cm²/h respectively (Meidana et al., 1998)
| Mean flux of Azone-pretreated skin: CW 0.75W/CW 2.25W/pulsed 2.25W are 0.0388±0.0105/0.0378±0.0766/0.0540±0.0124 pmol/cm²/h respectively (Meidana et al., 1998) |
Ultrasound and insertion force effects on microneedles based drug delivery: experiments and numerical simulation 2015

US transducer; hairless male Wistar rats skin 250-300g, aged 4-5 months
Franz diffusion cell; In vitro hairless male Wistar rats skin 250-300g, aged 4-5 months

1.1 0.1 CW (5 min) 5% v/v 24-33ml 5% v/v aqueous ethanol, 1.1ml aliquots were withdrawn every 30min within 5h to a microscope (×125) Liquid scintillation counter; thermocouple; a camera attached to a microscope
Receptor solution: 37 Liquid scintillation counter; thermocouple; a camera attached to a microscope (×125) Receptor

Mean ±S.D. & t test, P<0.0208 over 5h Mean flux of radiolabeled [³H]hydrocortisone under US treatment of 0/0.1/2 W/cm² are: 0.105±0.023/0.047±0.006/0.81±0.14 pmol/cm²/h respectively. For histological results see Fig. 3 in the original paper (Meidan et al., 1998)

Calcein
Sonicator; In vitro full-thickness skin from porcine ears
Custom-made vertical glass diffusion cells

MW: 623 Da Log K o/w= 1.56
DC: 10 (2 hours)

0.02 15 200mg calcein in 11ml PBS at pH 7.4 38 ml PBS at pH 7.4 Confocal microscope Room temperature n/a

There is no quantitative data in this research. However, it is using cross-sectional view of the skin (confocal images) to show the permeation of calcein with/without ultrasound treatment for 2 hours duration within a 20 µm depth. It suggests that some areas showed great increment of permeation of calcein after the ultrasound treatment while some areas did not (Polat et al., 2011)

Three separate US Systems; back and flank skin of female Yorkshire pigs
In vitro 0.02 7.5 DC:50 (length 5s) 0.2% w/v calcein in 2.5ml PBS 12 ml PBS, sampled at 2 h intervals between 18 and 26 h UV–visible spectrophotometer absorbance wavelength: 494 nm Room temperature 25°C Mean±S.D. over 8 hours

The ultrasound treatment time varies according to when the electrical currents reach 225/275/335 µA. The results show that permeability is not affected by either electrical resistance or frequency change. The scales of permeability for passive/LTRs/non-LTRs/total are 1×10⁻⁶/10⁻²/10⁻⁵/10⁻³ cm/h, respectively (Morimoto et al., 2005)
**Inulin**

<table>
<thead>
<tr>
<th>In vitro</th>
<th>0.041</th>
<th>CW (2 hours)</th>
<th>0.06</th>
<th>1mM calcein in 10ml PBS at pH 7.4</th>
<th>22 ml PBS, Sampled at 30 min intervals between 12 and 14 h</th>
<th>Spectrofluorometry excitation wavelengths: 488 nm</th>
<th>Room temperature</th>
<th>Mean± S.D. over 2 hours</th>
<th>After 12 hours of passive diffusion, ultrasound is in turn applied for 30min to each at 0.06, 0.12, 0.3, 0.06 W/cm² for a total 2 hours. The flux increments from a base 1.1×10⁻² nmol/cm²/h are 120, 8900, 23000,5100 folds, respectively (Mitragotri and Kost, 2000)</th>
</tr>
</thead>
</table>

| In vivo on the upper back of hairless SD rats of either sex, 200-300g | 1.0 | 3 | DC: 80 (5 min) | 0.22μCi/μL | Urine in bladder collected using catheterization every 15-30 min within 2h | Liquid scintillation counter | Room temperature | Mean± S.E.M. & Student’s t test, P<0.05 over 2 hours | Mean secretion rate of radiolabeled [³H]inulin shows 5 times higher in the US treated group (n=4) than in the controls (n=4), for detailed data see Fig. 2 in the original paper (Levy et al., 1989) |

| In vivo | 0.02 | 7 | DC: 50 length 5s | 10 μCi/mL | Urine in bladder measured every 30min for 5h | Mean secretion rate of radiolabeled inulin before/after the ultrasound treatment are 7.4×10⁻⁶ cm/h and 1.5×10⁻⁴ cm/h, respectively (Boucaud et al., 2001) |
|---------|-------|---|-----------------|-----------|-----------------------------------------------|-------------------------------|---------------------|-----------------------------|--------------------------------------------------------------------------------------------------------------------|

**In vivo** followed by ultrasonic gel; Glucosylated inulin in warm water

**MW: 5.0k Da**

**In vitro** male WBN/ILS-Ht strain hairless rats

**US** custom-built US transducer; 

**diffusion cells**
<table>
<thead>
<tr>
<th>Insulin</th>
<th>Flanged</th>
<th>In vivo</th>
<th>0.02</th>
<th>2.5</th>
<th>DC: 10</th>
<th>3ml of insulin</th>
<th>0.6ml blood</th>
<th>Thermocouple; biochemistry</th>
<th>Donor solution: 27,</th>
<th>One-factor ANOVA</th>
<th>Blood glucose level of control group is 12.60±2.07 mmol/l, the initial and after US treatment for 5.0W, 10.0W are:</th>
<th>P&lt;0.05 over 60 min</th>
<th>12.91±1.68 &amp; 11.22±1.71/12.20±0.60 &amp; 10.65±6.22/13.33±0.67 &amp; 7.46±2.95 mmol/l respectively (Park et al., 2007)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MW: 5.8k Da</td>
<td>Flanged</td>
<td>Plastic</td>
<td>hydrated</td>
<td>for 1 h</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cylinder was glued to</td>
<td>Hairless</td>
<td>rats</td>
<td>280±20g, aged 8-10 weeks</td>
<td>Sonicator</td>
<td></td>
<td></td>
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</table>
| 1 mm thick water tight standoff arranged between the axillary area of the pig and the array | In vivo Yorkshire pigs (100–140 lbs) | 0.1 | DC: 20 | 100 U/ml insulin filled in 1 mm thick water-tight standoff | 0.3 ml blood samples from the ear vein every 15 min for 90 min | Blood glucose monitoring System | Room temperature | One-factor ANOVA analysis, P<0.05 for both 60 and 90 min | Blood glucose level before experiment is 146±13 mg/dl (n=6); Control group increased 31±21 mg/dl (n=3) after 90 min; US treatment group decreased 71±5 mg/dl (n=3) from beginning at 60 min and decreased 91±23 mg/dl (n=3) from beginning at 90 min (Bommannan et al., 1992)

Note: CW = continuous wave; DC = duty cycle; PBS = phosphate buffered saline; S.E.M. = standard error of the mean; SD = standard deviation.
2.1.2.2 Thermal effect of sonophoresis

The most obvious phenomenon during the sonophoresis treatment is the thermal effect and it is particularly relevant when using high frequency. The absorption of the sound in skin increases as the ultrasound frequency goes up, which means that the energy would be stored in skin rather than transmit through (Boucaud et al., 1999). The rise in the temperature of the skin increases the kinetic energy of the drug molecules which have a positive effect on the drug diffusion rate. However, the intensity in the application of sonophoresis is usually low; therefore, the thermal effects on the kinetics energy of the drug molecule and hence the drug permeability is not significant. For example, when 1.5 W/cm² ultrasound was applied on hairless rat, the temperature change is found to be only around 1-2°C (Levy et al., 1989). If the temperature increases significantly due to ultrasound treatment, it can cause skin injury. In particular, it has been reported that when the temperature reaches 43°C and maintains this temperature for 60 minutes or longer, the cellular reproduction may be restrained (Kennedy et al., 2003). Although the thermal effect generated by ultrasound is the most basic phenomenon due to energy gain/loss, it has the potential to increase and control the drug diffusion rate when combined with MNs, particularly, dissolving MNs. We discuss this point further in section 2.1.4.

2.1.2.3 Convection in acoustic streaming

Another ultrasound related phenomenon is termed as acoustic streaming which is a kind of fluid flow driven by the pressure gradient and generated by acoustic field (Riley, 2001). The permeability enhancement due to acoustic streaming is hard to define but its importance is realized by the scientific community (Lavon and Kost, 2004). Different effects of the acoustic streaming can be identified by Reynolds number of the flow. With low Reynolds number, Lighthill (Lighthill, 1978) has described the relationship between the net force of unit volume \( F_j \) [N/m³] and forces that generated by the momentum flux \( \rho_0 u_i u_j \) (Reynolds stress) [N/m²], the pressure \( p \) [N/m²] and the viscosity \( \mu \) [N·s/m²]. The mathematical definition is given in equation 2.2.

\[
F_j = \rho_0 \left( \frac{\partial \bar{u}_j}{\partial x_i} \right) + \frac{\partial p}{\partial x_j} - \mu \nabla^2 \bar{u}_j \tag{2.2}
\]

Where \( \rho_0 \) is the density of the volume of the fluid [kg/m³], and \( u \) is the velocity vector [m/s]. As evident from the first two terms on the right hand side, the Reynolds stress and pressure
gradient affect the net force in the tissue positively (the term in left hand side of the equation). But, as shown by the third term on the right hand side, higher fluid viscosity results in lower net force which indicates that the ultrasound field can apply higher forces on the flow in a less viscous solution. Tachibana (Tachibana and Tachibana, 1993) shows that the diffusion of lidocaine under the same ultrasound condition is higher when it is in the aqueous formulation instead of a gel. Therefore, the permeability enhancement caused by ultrasound generated force was experimentally proven. The phenomenon was successfully extended by applying this mechanism on highly aqueous tissues. Lewis (Lewis et al., 2009) applied 1.58 MHz ultrasound on brain and avian muscle tissues, and report that the enhancements of Evans blue dye are 5.6 fold and 2.2 folds, respectively. Using the experimental set up in Figure 2.4, Cheung (Cheung et al., 2010) has demonstrated that under 3 MHz ultrasound treatment the permeability of bovine serum albumin (BSA) increases 1.6 fold in intrascleral delivery.

![Figure 2.4 The setup of ultrasound enhanced intrascleral delivery (Cheung et al., 2010).](image)

Although the researches on using acoustic streaming at low Reynolds numbers have shown increment on diffusion rate in soft tissues, the permeability enhancement on viable epidermis and dermis is still relatively unknown. As well known, viable epidermis and dermis of the skin are the most relevant skin layers for MN application as the MNs pierce the SC and deliver the drugs in the skin layers below the SC. However, it is logical to state that there are good potential for combining ultrasound with solid or hollow MNs because the MNs can create channels that reach viable epidermis. As such the ultrasound may reach the lower layers of the skin and help increase drug permeability in these skin layers. This combination of TDD
methods should be able to provide higher diffusion rate in comparison to sole MNs. There are some other mechanisms caused by acoustic streaming at high Reynolds numbers which are also important. These mechanisms will be discussed in sections 2.3.1.4-2.3.1.6.

### 2.1.2.4 Acoustic cavitation in high frequency sonophoresis

Acoustic streaming can produce different mechanisms in sonophoresis. The most important mechanism is acoustic cavitation (bubbles) which is generated in the liquid within or out of the skin. When ultrasound waves keep compressing and tensing the liquid in the tissue, the local pressure of liquid falls below vapour pressure, and therefore, the cavitation occurs. The cavitation can be divided into two types: stable and inertial discriminated by how long the bubbles can survive (Polat et al., 2011). Frequency, intensity and duty cycles are used to control the cavitation types to achieve different applications. The cavitation generated during high frequency ultrasound treatment is much smaller in size as compared to those from the use of low frequency ultrasound. The relationships between the frequency and the bubble radius are presented by Gaertner (1954):

\[
f(\varnothing) = \frac{1}{\sin^3 \varnothing \left[ -(\sin^4 \varnothing + \sin^2 \varnothing)(pR_0 + 3\alpha) - pR_0 \right]^{1/2}}
\]

\[
\varnothing = \arcsin \left( \frac{R_0}{R} \right)^{1/2}
\]

Where \( f \) is the frequency of the ultrasound [Hz], \( R_0 \) is the nucleus radius [m], \( \alpha \) (surface tension) is a constant related to the medium [N/m] and \( R \) is the radius of the bubble [m] under pressure \( p \) [N/m²]. The equation indicates that the ultrasound frequency \( f \) is inversely related to the radius of the bubble \( R \). The equation has also suggests that cavitation could be generated inside the skin or simply within the SC layer if the frequency is high enough.

### 2.1.2.5 Rectified diffusion

Due to the relationship between frequency and bubble radius, the most important phenomena in high frequency sonophoresis, which is called rectified diffusion (Figure 2.5), has been revealed by Blake (1949). The mechanism can be explained as follows. On the positive pressure half-cycle (the local pressure increased under the ultrasound field) the gas
in a small bubble will be compressed, the shell becomes thicker as a result the concentration gradient decreases because the bubble absorbs more drugs from surrounding environment. Some gas then diffuses outwards from the core of the bubble into the liquid. On the contrary, during the negative half-cycle of pressure, the surface of the bubble is expanded which makes it much larger than the compressed bubble. However, these two rates are not equal as the surface area of the bubble is greater during the negative (tension) half-cycle, and as diffusion rates are proportional to the exposed area, the bubble must gain some gas over a complete cycle (Neppiras, 1980).

Figure 2.5 A schematic diagram of rectified diffusion (Naji Meidani and Hasan, 2004).

In the process of the rectified diffusion, the bubbles generated by cavitation are pushed by the Bjerknes force under acoustic pressure gradient to move downward (Crum and Lawrence, 1975). The Bjerknes force is generated under the acoustic field which directly increases the diffusion rate of the drug molecules. It can then push the bubbles forward, thereby, increasing the diffusion rate. The basic expression of Bjerknes force is shown as:

\[ F = -\langle V \nabla p \rangle \] (2.5)

Where \( < > \) denotes the average over one acoustic period, \( V \) is the volume of the bubble and \( p \) is the acoustic pressure. This equation can be further extended to define the Bjerknes force at any location \( r \) and time point \( t \) (Louisnard, 2012):
\[ F_{Bi} = -G_i(r) \frac{1}{T} \int_0^T V(r, t) \cos(\omega t + \psi_i(r)) \, dt \] (2.6)

Where \( T \) is the acoustic period, \( V(r,t) \) is the instantaneous volume of the bubble, \( G(r) \) and \( \psi(r) \) are pressure and phase gradients, respectively. Because the rectified diffusion is the dominated effect in high frequency sonophoresis, the bubbles must be small enough to survive inside the skin. This means that the diffusion of molecules may not necessarily increase. Bommannan et al. (1992) have reported that after 20 min ultrasound treatment (2 MHz, 0.2W/cm²), the diffusion of salicylic acid (138.12 Da) is not increased. However, the diffusion rate is 4 times higher at 10 MHz 0.2W/cm² when the bubble size is small enough to move inside the skin. But, if the size of the bubbles is further reduced, the diffusion rate is dropped to 2.5 fold at frequency of 16 MHz and power of 0.2W/cm². Therefore, properly selected frequency will significantly increase the diffusion rate. This provides a great potential to deliver medium-sized or large molecules by using the micro needles and ultrasound combination.

2.1.2.6 Acoustic cavitation in low frequency sonophoresis

The low frequency sonophoresis generally refers to the ultrasound frequency between 20 and 100 kHz. Unlike high frequency sonophoresis, the low frequency sonophoresis research has only been introduced over the last 10 years (Mitragotri and Kost, 2004). Researches showed that low frequency ultrasound have much better effect on drug delivery enhancement (both low and high molecule weight drugs) than high frequency ultrasound (beyond 1 MHz). Mitragotri et al. (1996) have shown that the enhancement ratios induced by low-frequency ultrasound (20 KHz) is up to 1000-fold than that induced by therapeutic ultrasound (1 MHz) on butanol (74.12 Da) and sucrose (342.29 Da). This is due to the fact that inertial cavitation becomes the dominated mechanism and it can directly disrupt the SC layer to increase the permeability. The application of low-frequency ultrasound can be divided into two forms: simultaneous sonophoresis (decreased after ultrasound is turned off) and pre-treatment sonophoresis (remains in highly permeable state for several hours). The mechanisms of both of these two methods rely on inertial cavitation, i.e., bubbles generated within the coupling medium by ultrasound and would grow and collapse violently. The difference between them is the pre-treatment sonophoresis and generates more bubbles to change the structure of the stratum corneum (about 30% of the lipids layer is removed by
micro-jet (Alvarez-Román et al., 2003)) while the simultaneous sonophoresis only intends to increase the porosity.

As mentioned before, the main type of cavitation which helps in permeability increment is the inertial cavitation (Merino et al., 2003). It occurs due to pressure variations induced by ultrasound, resulting in rapid growth and collapse of bubbles formed in the coupling medium. The collapsing of the aforementioned bubbles in a spherically symmetric environment results in the release of a shockwave causing structural changes of the surrounding tissue. The suddenly raised pressure gradient conducted by the shockwave spreads evenly on the skin surface. Therefore, it can only produce limited damage. However, the skin surface will cause the bubbles involutes asymmetrically which results the bubbles collapse from the top surface (Lauterborn and Ohl, 1997). Water is then forced into the bubbles and a high-speed micro-jet will be developed from the top surface. The power generated by the micro-jets transmits downward and is focused on a tiny spot of skin surface. These micro-jets can severely disrupt the SC layer and have been confirmed as the main contribution to the permeability increment (Wolloch and Kost, 2010). The mechanism of forming micro-jets is shown in Figure 2.6.

![Figure 2.6 The mechanism of cavitation collapse near skin surface which creates a micro-jet](Metropolitan Acoustics, 2014).

Because of the huge bubble size in low frequency sonophoresis, the main effect is based on the microjet that occurs during bubbles collapse. The permeability can remain high for hours after a short ultrasound application. Therefore, a relatively high input power must be employed to create channels on the stratum corneum otherwise the permeability cannot be maintained. Figure 2.7 indicates that insufficient power of inertial cavitation do not create significant pathway on the porcine stratum corneum by micro-jet.
The inertial cavitation has been studied by many researchers. It shows excellent potential when combined with solid MNs (discussed in section 2.1.4). Solid MNs can provide opportunities for the inertial cavitation to physically contact the viable epidermis. It will greatly reduce the pretreatment time and keep the permeability increased for a longer period of time. These issues are discussed in detail in the following sections.

2.1.2.7 Safety of ultrasound application

The tolerant limit of skin to the ultrasound is an important issue for the applications of sonophoresis. However, only a few studies have been conducted in this area and it seems that this technology is still underdeveloped and has a long way to go before clinical trials. An in vitro study on human skin shows that at 2.5W/cm$^2$, the skin structure modification can be identified under electron microscopy (Boucaud et al., 2001). Although these is no in vivo study on human, ultrasound has been applied on canine subjects and urticarial reaction is found when the power reaches 16W (Singer et al., 1998). Currently, the intensity of most sonophoresis applications are under 3.5W/cm$^2$ (Ahmadi et al., 2012), so that the safety can be assured.

2.1.3 Microneedles in TDD

The MNs research has been carried out for over 40 years now and it is one of the most promising technologies among the TDD methods. MNs are a technology developed from transdermal patches and hypodermic needles, attempting to gain advantages and eliminate disadvantages from both. The idea of MNs comes from the patent of Gerstel and Place (United states Patent No. 3,964,482, 1976) in the early 1970s when they introduced the concept to make micropores in the skin. In the 1990s, the microchip fabrication technology provided the new way to make longer three-dimensional microstructures of silicon and mass production of microfabrication tools so that the experimental demonstrations can be made. The first study of using MNs to enhance TDD process was devoted in 1998 (Henry et al., 1998). Following this work, MNs technology has been developed rapidly and greatly extended in pharmaceutical area. Compared to hypodermic needle, MNs are minimally invasive and can significantly reduce the pain depending on the length of the needle. Gill et al. (2008) have used different length of MNs from 480 µm to 1450 µm tested on human volunteers and found out that the needle length below 750 µm is painless and bloodless. However, the MNs that are less than 300 µm long have been shown not able to penetrate the skin (Mukerjea et al., 2004). The main development of the MNs technology which makes it distinct from other TDD methods is that it has greatly extended the range of drug
molecular weight that can be delivered. Verbaan (2007) used 700 µm MNs array to successfully deliver fluoresceinisothiocyanate coupled dextran which has molecular weight of 72 kDa. Influenza vaccine has also been delivered by using biodegradable MNs on mice (Sullivan et al., 2010).

The MN research has developed strong activities in the last decade as indicated by a large number of publications. The average increment rate of MNs publications is 77.9% per year for the past 10 years (2004-2013) while the overall increment rate is 14.9% since 1970. To have a better understanding on the developing future of MNs, an ARIMA model is employed again which provides a rough forecast on the trend of MNs research. The data for the number of publications have been differenced twice to acquire a relatively stable model. The algorithm orders that are suitable for this model are chosen by considering the trend of autocorrelation coefficient and partial autocorrelation coefficient calculated from the model. As a result, the AR (1) and MA (1) models have been confirmed as the optimum choice with minimum deviation from the original data (mean absolute percent error 139.8%). The trend of MNs research until 2020 is then forecasted. After recognizing the pattern of the past 40 years, the ARIMA model suggests that the number of publications will reach 694 in 2020 in comparison to 446 in 2013 which gives a 99.2% increment rate per year. The observed, fitted model and forecasted data have been shown in Figure 2.8 which suggests that in comparison to the trend in the past 10 years, the research in the MN area will become more active in the coming years.
Figure 2.7 The cavitation generated by micro-jet applied on the porcine stratum corneum. A) No ultrasound applied on the skin; B) 20 kHz, 2.4 W/cm² ultrasound applied on the skin for 10 min (Tezel and Mitragotri, 2003).

There are a number of review papers focused on MNs related areas other than the mechanisms of MNs, for example fabrication techniques of MNs (Nguyen et al., 2013), permeability enhancement of MNs (Al-Qallaf and Das, 2009) and ethical study of MNs (Olatunji et al., 2013) etc. However, this review intends to explore the potential of MNs combined with sonophoresis. Therefore only a brief discussion on MNs is presented. Conventionally, MNs is divided into two different types: solid MNs and hollow MNs and their main mechanisms are shown in Figure 2.9.

Figure 2.8 The observed, fitted model and forecasted trend of MNs research presents from 1970 to 2020 using ARIMA model.

This classification method can only present the basic idea of the delivery process. The real delivery efficiency depends on many other factors. Martanto et al. (2006) performed experiment to reveal the relationship between the MNs insertion depth and force. They also found out the real insertion depth is much lesser than the length of the needles due to the skin buckling. Their histological results shows the real penetration is only 100-300 µm by applying 1080 µm length MNs.
The geometry of MNs has been proved to be another important parameter that can directly affect the diffusion results (Davidson et al., 2008). Parameters such as tip radius, density, distance between needles etc. are all brought to the consideration for being the important factors in MNs patch design (Olatunji et al., 2012). Besides these factors, the main purpose of MNs enhanced TDD is to reduce the resistance of the skin. Therefore, high force has been applied to reduce the skin buckling effect so that it can increase the real insertion depth (Cheung et al., 2014). Super short MNs with 70-80 µm length are also introduced to increase the permeability (Li et al., 2010). This kind of MNs is not aimed at penetrating the skin but to scrape the skin surface in order to lessen the thickness of SC layer.

2.1.3.1 Solid microneedles

The solid MNs are stiff and steady in structure and deliver drug by coated drugs on their surface or micro conduits created on epidermis to let drugs go through it. The methods of solid MNs delivery can be done in four different ways:

Figure 2.9 Main mechanisms of applying MNs: A) Solid MNs using poke with patch method; B) Solid MNs using coat and poke method; C) The Dissolving MNs and D) Hollow MNs for liquid delivery (Escobar-Chávez et al., 2011).
(1) The poke with patch approach was proposed by Henry et al. (1998) who used solid MNs to penetrate human cadaver skin and reported that the permeability of calcein increased by up to three orders of magnitude. The mechanism of poke with patch method involves the use of solid MNs to pierce the stratum corneum first and then put a patch during the insertion or immediately after removal of the needles. An image is shown in Figure 2.10 to illustrate this point.

![Image](image_url)

**Figure 2.10** a) Solid MNs penetrated into the skin b) after the MNs are removed, the treated skin is ready for applying the drug loaded patch (Verbaan et al., 2007).

Research done by Wermeling et al. (2007) indicated that Naltrexone (used to treat opiate and alcohol addiction) only takes two hours to reach the steady state plasma concentration with MNs pre-treatment and last for 48 hours while the transdermal patch cannot give any detectable results over 72 hours. Martanto et al. (2004) compared the hypodermic needle with the solid MNs (105 needles) by injecting insulin to diabetic rats and reported positive results on delivering large MW proteins.

(2) The coat and poke approach is used to coat the drug on the surface of the MNs which is then applied on the skin (Figure 2.11). Large molecules such as human growth hormone (22 kDa) and ovalbumin (45 kDa) can be transported through skin (Daddona et al., 2007) in this way.
The main advantage of this method is that the MNs can retain its mechanical strength while delivering drugs. Therefore, the permeability loss during the skin recovery can be avoided (Maaden et al., 2012). However, the drug reloaded during the delivery process can be a problem. The most efficient way is to put the matrix of MNs on a roller with coating device on one side. So the MNs can maintain in inserting and coating rotation when rolling on the skin surface. But this method cannot increase the diffusion rate after the drug is injected which makes it faces the same limitation with the poke with patch approach.

(3) The dip and scrape method employs the array of MNs coated with drugs to treat and then scrape multiple times across skin to create micro-abrasions. These micro-enhancers have proved that they can effectively breach the skin barrier and increase the permeability of the drug molecules (Mikszta et al., 2002).

(4) The dissolving MNs are made from a polymeric material and will release drugs after the MNs dissolves within skin (see Figure 2.12 for an example). The main problem in this kind of MNs is the drug mutations during the high temperature moulding and fabrication (Donnelly et al., 2009). A novel dissolving MNs introduced silk fibroin as the base of the MNs because it is rigid in structure, friendly to donor permeates and dissolving very quickly (You et al., 2011). More recently, biopolymers have been used in the fabrications of dissolving MNs because they can reduce the cost of material and also increase the biocompatibility of the products (Olatunji et al., 2014).

Besides, there are new forms of MNs being presented in order to maximize the drug deliver rate, e.g., swelling MNs. This kind of MNs combines drug molecules with swelling polymer.
which will imbibe interstitial fluid of the skin during insertion and increase the contact area between drug solution and epidermis (Donnelly et al., 2014).

2.1.3.2 Hollow microneedles

The hollow MNs have similar mechanism with hypodermic needles but they are much smaller in size (e.g., 500 µm in length). The drugs can continuously flow into the skin through the hollow capillaries but in a low transmission rate. If the pressure is high the drug would overflow through the bypass between needle and skin to the atmosphere. One significant advantage of the hollow MNs among other types is that it can extract a small amount of blood sample underneath the skin which enables monitoring on quantities of body fluid, for example, blood glucose level (Smart and Subramanian, 2000). This technology is then further developed by optimising the geometry and arrangement of the patch so it can give reasonable extraction rate (Li et al., 2013). Due to the small size of the hollow MNs which increase the difficulty of the penetration and injection, works also have been done on how to optimise the process of MNs insertion and injection pressure during drug delivery. Martanto et al. (2006) demonstrated that a little retraction during injection process can significantly increase the fluid infusion because it releases the compaction of the skin. The flow rate inside the hollow needles is another parameter that has been studied and optimised (Stoeber and Liepmann, 2005). However, the blockage of fluid inside the hollow MNs which comes from the resistance imposed by dense dermal tissue is still need to be solved (Martanto et al., 2006). Besides the above, the stiffness of the MNs, difficulties in fabrication and cost are very important issues in the development of hollow MNs (Lhernould, 2013).
2.1.4 Potential of combining microneedles and sonophoresis

This review focuses on discussing the ultrasound and MNs combination as a TDD method for the future. Therefore, we devote this section of this review to discuss this point in detail. As discussed in the review before, research on MNs and ultrasound are both actively pursued in recent years, which involve different mechanisms for drug delivery and transport. Therefore, the main point of discussion in this section is how to combine these mechanisms. In addressing this issue, we discuss the different possible ways and benefits of combining MNs and ultrasound.

We can use hollow MNs to pierce the skin and apply ultrasound simultaneously, where the hollow MNs should be under the ultrasound field. The hollow MNs are able to provide certain permeability increment while the simultaneous ultrasound field can enhance the flow rate via convection (this combination can be referred to the categories (iv) and (v) in Figure 2.1). In fact, there are already some attempts to combine these two mechanisms. For example, a Singaporean research group has attempted this combination to deliver calcein and BSA (Chen et al., 2010). They have used 100 µm long hollow MNs (80 µm outer diameter at the
base) to pierce the skin and have attached an ultrasound transducer at the back of the MNs patch. The ultrasound output parameters have consisted of 20 kHz frequency, 0.5W/cm² intensity and 20% duty cycles (length 10µs) which can maintain the temperature stable at 37°C. The enhancement of this specific circumstance shows 9 times higher for calcein and 12 times higher for BSA in compare to passive diffusion, respectively, as shown in Figure 2.13.

We can also use solid MNs combine with ultrasound cavitation. The solid MNs can create visible holes that will provide permeability increment. If we apply high intensity ultrasound on the MNs pretreated base, the ultrasound cavitation will be able to produce more significant enhancement than on its own. This is because the ultrasound cavitation can contact the underneath layers of the skin where these bubbles can release more potentials. This combination is especially efficient in the delivery of large molecules and it can be a good preparation for a long-term TDD (this combination can be referred to the categories (iii) and (iv) in Figure 2.1). A recent paper which has used this kind of combination to delivery BSA through skin is reported by Han and Das (2013). Two sets of solid MNs with length 1.1 mm and 1.4 mm were applied for 10 min to create pores and disrupt the skin surface. The 20 kHz, 9-18W ultrasound is then mounted to create inertial cavitation for another 10min. The permeability increment results of the combination are shown in Figure 2.14.

![Figure 2.13 BSA release profile using simultaneous ultrasound combined with hollow MNs (Chen et al., 2010).](image-url)
Figure 2.14 BSA permeability results of passive diffusion, ultrasound only, MNs only and MNs combined with ultrasound (Han et al., 2013).

The data from Figure 2.14 shows a significant increment of permeability that is corresponding to the ultrasound power at 15W. It indicates the cavitation effect reaches its maximum performance when proper ultrasound output is applied on the target area. According to equation 2.3, the size of the bubbles generated by the transducer at certain power and frequency should remain constant during the process. Therefore, the optimal ultrasound output should be not only sufficient to initiate the inertial cavitation, but also not too strong to wreck the bubbles before the micro-jets can be produced.

It may also be possible to use solid MNs to pretreat the skin and then apply ultrasound simultaneously. This combination can be specialized to deliver medium size molecules. The difficulty in the delivery of medium size molecules is the low diffusion rate after the molecules have entered the viable epidermis. Solid MNs are sufficient to deliver the medium size molecules to the underlying layer. The simultaneous ultrasound can then apply pressure gradient on the molecules and achieve diffusion increment via rectified diffusion (this combination can be referred to the categories (iv) and (v) in Figure 2.1).

Furthermore, we can use dissolving MNs to pierce the skin and then apply low intensity simultaneous ultrasound to active the maximum thermal effect. The dissolving MNs can pierce into skin and release drug at a constant rate according to the local temperature. Ultrasound is able to provide relatively accurate local temperature adjustment. It can provide temperature modification thereby controlling the drug release rate. If this combination can be
well developed, it will achieve both drug delivery rate increment and control (this combination can be referred to the categories (ii) and (v) in Figure 2.1).

We can use high intensity ultrasound to pretreat target area and then apply dip and scrape MNs or super short MNs to deliver drugs. The inertial cavitation can reduce the resistance of SC layer so it will be easier for the MNs to create micro-abrasions on skin surface. This combination can provide a simple way to apply drugs that need rapid onset at topical area. The thermal effect comes along with the high intensity can also increase drug absorption rate (this combination can be referred to the categories (iii), (iv) and (v) in Figure 2.1).

Although the MNs combined with sonophoresis methods show significant prospects, the consideration of MNs combined with other TDD technologies are also equally promising. Therefore, a cluster analysis has been employed in this work to reveal the current trends on these cooperating researches. The analysis is able to make suggestions on how these trends are likely to develop in the future.

![Figure 2.15 Cluster analysis for determining the significance of current research on combined MNs with other TDD technologies.](image)

We have used two specific concepts to design the clusters: the research activity and level of contribution from each research activity. The research activity is indicated by the number of publications of each year divided by the mean of the total number of publications of that subject group. The contribution level for each subject is defined as the number of publications of each year divided by the total number of publications of MNs of the same
year. As the MNs papers cannot compare to themselves, the values of the contribution level of MNs are stochastically spread across the x axis. The four subjects are then plotted as four clusters in different shapes and colours (Figure 2.15). The k-mean clustering method (Cheng, 1995) is adopted to calculate the centroid of each cluster by measuring their squared Euclidean distance. The results of the cluster analysis (Figure 2.15) indicate that both ultrasound and iontophoresis have shown relatively high research activities and levels of contribution. Specifically, the ultrasound combined with MNs shows the highest potential for research activity which suggests that this combination is developing fast in the past 10 years and more researches will be conducted in the future if the current trend continues. The iontophoresis and electroporation combined with MNs shows the highest contribution level which indicates that this is the research activity where the most current combinational researches are focused on.

2.2 Modelling and simulation of microneedles systems

2.2.1 Background

MNs is a technology developed based on the concept of having a hybrid between hypodermic needles and transdermal patches. It is designed such that rather than a single long needle penetrating all the way into all the layers of the skin and causing pain as in the case of a hypodermic needle (Prausnitz, 2004), it is consisted of micro-scaled needles (Henry et al., 1998) which are not long enough to trigger the nerves in the dermis but capable of helping drug molecules to bypass the outer layer of skin, namely the stratum corneum (Prausnitz, 2001). Nowadays, MNs has become one of the most efficient methods in the field of transdermal drug delivery (TDD) and its enhancement on the rate of TDD has been experimentally proven since 1998 (Henry et al., 1998). The MNs research is mainly focused on two areas:

(i) Experimental aspect, researches dedicated to investigating the use of MNs as a tool to increase the permeability and controlled release of different drug molecules, vaccines and micro-particles through skin (Pierre and Rossetti, 2014). To understand the efficiency of MNs under different circumstances (drug molecule properties, skin conditions, force applied on the MNs etc. (Cheung et al., 2014)), TDD experiments have been conducted to demonstrate the relationship between the diffusion profiles and those parameters.

(ii) MNs fabrication technologies, such as micro-electro-mechanical systems (MEMS) (Indermun et al., 2014), laser cutting (Aoyagia et al., 2007) and lithography
(Pérennès et al., 2006). These technologies will enable the MNs to be fabricated using different materials and diverse geometries of the MNs can be achieved in a mass reproducible way.

A statistical study of the papers working on different areas of MNs research is shown in Figure 2.16. From the chart it is apparent that the bulk of the research on MNs focuses on experimental methods on transdermal and in vivo studies and fabrication of MNs, thus indicating that there is a need to focus more research effort on modelling. MNs related modelling is an important area where the theory can be applied but the research attention is inadequate. Focusing more research effort toward prediction of MNs performance via modelling could help direct overall research effort towards key areas which need addressing. A review of the existing mathematical models is therefore important to bring together the work done so far on MNs and identify the key areas which need further focus.

![Figure 2.16 A statistic study showing the distribution of MNs papers on different areas (Scopus as the search engine) (Scopus, 1995).](image)

The first work reported on MNs modelling was in 1999, the work looked at modelling fluid extraction using hollow MNs (Brazzle et al., 1999). The possibility of developing MNs modelling towards achieving optimization and testing without running any experiment is rather attractive. This can save money and time by eliminating need for time consuming and expensive experiment and fabrications. This chapter aims to present the current states of the MNs modelling technology and discuss some future prospects. To this effect, we will review
modelling approaches in areas of design, transdermal delivery, skin insertion and MNs forces.

2.2.2 Microneedles modelling

The modelling of MNs is a transitional stage that converts the MNs design into computational model which will be then used to run simulations. A mathematical model requires that the model completely matches the designed MNs and has the details of the design included if the geometry is complicated because any change of the geometry can lead to different simulation results (Al-Qallaf et al., 2007). After the computational domain is created, the properties of both MNs and MNs treated skin need to be properly selected following the required conditions in the design stage. The former properties are including the mechanical properties of the MNs structure (Young’s modules, Poisson’s ratio, ultimate tensile strength etc.) for insertion studies (Loizidou et al., 2015) and properties of the drug solution to characterize the parameters for diffusion. The latter properties are related to the permeation studies which will affect the diffusion profiles of the drug molecules in the MNs treated skin model (Gomaa et al., 2012) and the deformation of the skin due to MNs insertion (Groves et al., 2012; Boonma et al., 2013).

2.2.2.1 Parameters of microneedles modelling

The dimensions of the designed MNs will be converted into input parameters for modelling so the created computational domain is eligible for further simulation. The generated domain is a substitute of the designed MNs in the computer where different tests can be achieved within that domain, i.e., a computational domain is created in computer program ‘ANSYS’ which is shown in Figure 2.17:

Figure 2.17 An example of designed hollow MNs modelled in details (Aggarwala and Johnston, 2004).
All the parameters involved to describe the MNs geometry are input to the program and the alignment of those MNs are also provided for modelling process. For the parameters that describing MNs' geometry and arrangement on the MNs patch, there are five key aspects which have been analyzed and discussed: the penetration depth/MNs length, both tip and base diameter of the MNs, center to center spacing between MNs, Numbers of MNs in the array and the shape of the MNs (square, diamond, triangle, rectangle or special designed (Al-Qallaf and Das, 2009)). However, these parameters are not individually affecting the diffusion but connected as a synergetic system. Therefore, new parameters are introduced to define the relationships between those MNs parameters, thereby describing the system more properly for the purpose of optimization. For example, a parameter called aspect ratio of the pitch (α) is selected to define the relationship between the two key parameters: MNs radius R and center to center spacing Pt (pitch) (Al-Qallaf and Das, 2009). It describes the ratio of the pitch over MNs radius so the arrangement of MNs on the patch can be confirmed according to the conditions that are limited in the design stage. A schematic illustration of square MNs array is shown in Figure 2.18.

![Figure 2.18 An illustration of using the parameters Pt and R to describe the arrangement of MNs on the patch (Al-Qallaf and Das, 2009).](image)

The parameters in Figure 2.18 are crucial for extending the modelling from single MNs to multiple MNs array in order to analyze the efficiency of the whole patch.

Following the definition of the MNs geometry and arrangement, there are other parameters need to be input accordingly for different purposes of simulation. For instance, solid MNs has higher stiffness than hollow and dissolving MNs. Therefore, the simulation will be mainly focused on the skin deformation during insertion rather than the deformation of MNs itself. In
this case, the deformation of the solid MNs will be ignored during the analysis of MNs insertion, thereby only the mechanical properties of skin will be imported.

Figure 2.19 Modelling for the deformation of skin penetrated by solid MNs (Boonma et al., 2013).

The Figure 2.19 shows the simulation of skin penetrated by solid MNs which is considered as an independent entity for the whole insertion process. Only the deformation of skin is recorded and the modified domain will be then employed for conducting further simulations. The dissolving MNs consists of polymers is relatively soft comparing to the solid MNs. Hence, the mechanical properties of the MNs are also imperative to be measured in order to optimize the formulation of the MNs and the maximum load it can bear. An example model of dissolving MNs consists of carboxymethylcellulose and maltose (CMC/MAL) is shown in Figure 2.20.

Figure 2.20 A) the CMC/MAL dissolving MNs is piercing skin sample under 5N load; B) the buckling force is predicted based on the mechanical properties of the MNs (Loizidou et al., 2015).

Comparing to the solid MNs, the consideration of the mechanical properties for the dissolving MNs is crucial to avoid circumstance such as the MNs is broken before the skin is fully penetrated.
After the original domain is altered using the previous parameters, the adjusted domain is then ready for permeation studies which require information of the related drug molecules. Parameters of the drug molecules that can affect the diffusion coefficient such as molecular weight, partition coefficient can be used to characterize the diffusion profiles in the simulation. Gomma et al. (2012) selected a series of six structurally related ionic xanthene dyes with diverse molecular weight to run the simulation on MNs treated porcine ear skin. The molecular weights of those six dyes are ranged from 366.80 Da (Rh 110) to 10,000 Da (RITC-D). The results of this study confirmed the molecule weight is the most significant factor that affects the diffusion rate. The cumulative amount of those six dyes through MNs treated porcine skin are compared in Figure 2.21.

![Figure 2.21 The Cumulative amount of six dyes permeated through MNs treated porcine skin (Gomma et al., 2012).](image)

2.2.2.2 Programs for microneedles modelling

There are different kinds of computer programs that have been occupied for MNs modelling including commercial software, in-house programs, and programs designed in commercial software. The in-house programs are usually developed for special purposes and more efficient when solving problems under the designated circumstances. For instance, when the aforementioned parameter ‘aspect ratio of the pitch (α)’ needs to be tested for the optimization of squared MNs patch, an in-house java program is developed to achieve the requirements. The interface of the program is shown in Figure 2.22.
Figure 2.22 The interface of the java program for optimizing the arrangement of MNs on a square patch (Al-Qallaf and Das, 2009).

The best ratio is then calculated so that other researchers can use this ratio to optimize their design accordingly with great continence. Based on the optimized parameters acquired from the program, the permeability results of different drug molecules through MNs treated skin can be deduced by using the related diffusion coefficient. There are permeability results of some sample molecules (calcein, insulin, bovine serum albumin, nanosphere particles with radii of 25 and 50 nm, respectively) are presenting in Figure 2.23.
Figure 2.23 Relationship between the permeability and diffusion coefficient of the optimum solid (n=20, R=19 μm and A=0.04 cm²) and hollow (n=13, R=140 μm and A=0.53 cm²) MNs (Al-Qallaf and Das, 2009).

MATLAB (Moler C, 1970) is another commercial software that can provide numerical computing environment for user to do the programming. It has been widely used to achieve different aims. A recent study used MATLAB to acquire pore profiles from histological images that showing the cross-sectional view of the MNs treated skin (Han and Das, 2015). The computational domain in this study is directly obtained from the histological images of MNs treated skin instead of using software to create the domain based on the shape of MNs. This is a new paradigm of MNs modelling because the computational domain is directly acquired from the real histological, thereby the actual experimental conditions are also contained in the domain, for i.e., the insertion forces, insertion durations and real insertion depth. This method can provide more information for the modelling so the accuracy of simulation results can be improved. The mechanism of this program is shown in Figure 2.24.
Figure 2.24 The histological image of sliced skin is processed by MATLAB program. A) The original image; B) The outline of the skin has been captured; C) Simulated diffusion profile of the target drug molecule (Han and Das, 2015).

The simulated results of insulin are compared with experimental results under same conditions (porcine ear skin, 1.4mm solid MNs, same initial concentrations and time duration) which is shown in Figure 2.25. The concentration profiles for 4 hrs in the receiving compartment for both experimental and theoretical results indicate a relatively good comparisons. This confirms the idea of using histological image for MNs modelling is reasonable and has a very promising future.
Figure 2.25 Numerical simulation data compared to the experimental results. The concentration profiles of insulin are presented (Han and Das, 2015).

MATLAB can also be used for simulating the trajectories and penetration depth of micro-particles delivery by gene gun (Zhang et al., 2015) as mentioned previously. The penetration profile of the micro-particles through different skin layers are shown in Figure 2.26.

Figure 2.26 The overall view of the micro-particles trajectories delivered by gene gun (Zhang et al., 2015).

The penetration depth of the micro-particles through MNs treated skin is then calculated and the outcomes are reasonable comparing to the experimental results (Figure 2.27).
2.2.3 Microneedles simulation and optimization

2.2.3.1 Systematic simulation of the microneedles enhanced TDD

After the MNs design is converted into computational model, the MNs enhanced diffusion can be conducted based on those imported parameters. However, the holes created by MNs are more often chosen for simulation instead of the MNs model itself as those holes are directly connected to the skin. Therefore, a complete simulation of TDD using MNs can be considered as a system in which the MNs model is just one crucial component. The system is conventionally consisted of three components: the MNs model (or the hole it created), the skin and blood stream but can always be extended for higher accuracy. For example, the three components system for pharmacokinetic study can be expressed as equation (1)

\[ \text{Model results} \]

\[ \text{Experimental results} \]

Figure 2.27 The effect of the micro-needle length on the penetration depth of the stainless steel micro-particles (30 μm) (n=3) (Zhang et al., 2015).

The diffusion profile in the computational domain is generally simulated using commercial software that based on finite element method (FEM), such as ANSYS (Aggarwal and Johnston, 2004), COMSOL (Han and Das, 2015), Preview (Groves et al., 2012) etc. After the designed models are imported into those software, the diffusion results will be calculated following the configurations input by users.
which assumes the cells in the skin are ‘bricks’ that simply blocking the drug molecules and reducing the diffusion rate (Al-Qallaf et al., 2007):

\[ V_b \frac{dC_b}{dt} = \left( \frac{dQ}{dt} \right) S_a - K_e C_b V_b \]  

(2.7)

Where \( C_b \) is the concentration in the blood, \( V_b \) is the volume of distribution in the blood, \( K_e \) is the rate of elimination in the blood, \( dQ/dt \) is the flux through the skin surface and \( S_a \) is the contact area of the skin surface. This equation is sufficient for most of permeation studies in TDD. However, there are special circumstances where skin cells should be considered as ‘sponge’ instead, i.e., drugs that can be metabolized in the viable epidermis or lipophilic compounds that are bonding to the skin cells redefine all the related cells as a new entity in which each cell represents a small reservoir, hence it requires adding a new component into the simulation system. This new component interacts with the drug solutions from both skin surface and blood circulation which is described by equation (2.8) and (2.9) (Al-Qallaf et al., 2009):

\[ V_b \frac{dC_b}{dt} = \left( \frac{dQ}{dt} \right) S_a - (K_e + K_{12}) C_b V_b + K_{21} C_t V_t \]  

(2.8)

\[ V_t \frac{dC_t}{dt} = K_{12} C_b V_b - K_{21} C_t V_t \]  

(2.9)

Where \( K_{12} \) and \( K_{21} \) are the transfer rate between skin reservoir and blood circulation, \( C_t \) is the concentration in the skin reservoir and \( V_t \) is volume of distribution in the skin reservoir. Although the pharmacokinetic model is able to display the mass transfer status of all components in the system at both transient and steady states, but the parameters such as \( K_{12} \), \( K_{21} \) and \( K_e \) are difficult to be quantified. Therefore, the blood stream is usually assumed to have 100% absorption on drug solutions to avoid the complexity and back diffusion in the skin is also ignored.

### 2.2.3.2 Simulation of the microneedles treated skin

The skin performs the main obstruction between the drug formula and blood stream. The diffusion of drug molecules are hindered, thereby causing concentration gradient in the skin. The diffusion profile of drug solutions in the MNs treated skin can be simulated using Fick’s law in which diffusion coefficient is employed to describe the concentration variation. The steady and transient state of diffusion should apply Fick’s first (equation 2.10) and second law (equation 2.11), respectively.
\[ J_{ss} = -D \frac{dC}{dx} \quad (2.10) \]

\[ \frac{\partial C}{\partial t} = D \frac{\partial^2 C}{\partial x^2} \quad (2.11) \]

Where \( J_{ss} \) is the flux, \( D \) is the diffusion coefficient, \( C \) is the concentration in the skin, \( x \) is the depth from top surface of the skin and \( t \) is the time. The diffusion coefficient which is related to the properties of the skin and size of the drug molecules can be calculated using Stokes-Einstein equation (equation 2.12) when the skin is considered as isotropic material (McAllister et al., 2003).

\[ D = \frac{k_B T}{6\pi \eta r} \quad (2.12) \]

Where \( k_B \) is the Boltzmann's constant, \( T \) is the absolute temperature, \( \eta \) is the viscosity of the skin and \( r \) is the radius of the drug molecules. However, the skin has different layers and complicated inner structures which make the value of diffusion coefficient calculated from equation (2.12) inaccurate. Based on the special conditions of skin, three methods are introduced in order to increase the accuracy of the calculations: (i) the diffusion coefficient can be deduced from the partition coefficient in consideration of skin has multilayer structure. The values of the diffusion coefficient are different for each layer of the skin so they should be calculated separately using the equation (2.13) (Hansen et al., 2008).

\[ D = -\frac{J_{ss} h}{K_{i/j} c_0} \quad (2.13) \]

Where \( K_{ij} \) is partition coefficient between different diffusion compartments (donor compartment, SC layer, viable epidermis and dermis), \( h \) is the thickness of the skin layer and \( c_0 \) is the initial concentration of the skin layer. (ii) The equation (2.13) provides diffusion coefficients for every layers in the skin. To further explore the properties of those layers, the skin is considered as porous material in which tortuous channels are existed as pathways for the transportation of drug molecules. While the drug molecules are inside those channels, the hindrance factor \( H(\lambda) \) also needs to be included where \( \lambda \) is the ratio of molecule radius over pore radius. Therefore, the size of the molecules will decide the effectiveness of the hindrance factor, i.e., equation (2.14) shows the hindrance factor when the \( \lambda \) is less than 0.4 (Olatunji et al., 2012).
\[ H(\lambda) = (1 - \lambda^2)(1 - 2.104\lambda + 2.09\lambda^3 - 0.095\lambda^5) \] (2.14)

After the hindrance factor is calculated, it will be imported into Fick's law along with the porosity and tortuosity of the skin which has been shown in equation (2.15) and (2.16) (Kushner et al., 2007).

\[ J_{ss} = -\frac{\varepsilon}{\tau}D^\infty H(\lambda) \frac{dC}{dx} \] (2.15)

\[ \frac{\partial C}{\partial t} = \frac{D^\infty H(\lambda)}{\tau^2} \frac{\partial^2 C}{\partial x^2} \] (2.16)

Where \( D^\infty \) is the diffusion coefficient of the drug solution at infinite dilution, \( \tau \) is the tortuosity of the channels, \( \varepsilon \) is the porosity of the skin. (iii) The third method is to acquire the diffusion coefficient via experiment where the time lag (the time duration when the diffusion reaches its steady state) needs to be measured. Although this method considers the skin as uniform material, but the diffusion coefficient is still more accurate than calculated from equation (2.12). The theoretical relation between the diffusion coefficient and the time lag is shown in equation (2.17) (Crank, 1975):

\[ D = \frac{l^2}{6t_{lag}} \] (2.17)

Where \( t_{lag} \) is the time lag and \( l \) is the thickness of the skin. There are also studies working on minor factors such as back diffusions in the skin which will not be further elaborated in this chapter (Morofuji et al., 2013).

### 2.2.3.3 Simulation and optimization of microneedles in the system

The MNs is functioning as the drug delivery vehicle in the system where all the alterable parameters are included, hence most of the optimization works will take place in this section. The parameters that related to the geometry and alignment of MNs have been mentioned in the section 2.2.3.1. However, those parameters must be combined with certain types of MNs to complete the simulation.
2.2.3.3.1 The simulation of solid microneedles

The solid MNs can be either coated with drugs while insertion (coat and patch) or used to create pores on the skin where drugs will be applied after the MNs are removed (poke with patch). The poke with patch method has more capacity for drug loading so the pore created by MNs should be considered as reservoir for drug solutions. The permeability $k$ inside the reservoir can be described by equation (2.18) (McAllister et al., 2003).

\[ k = f \frac{D}{L} \]  

(2.18)

Where $f$ is the fractional area of the pore over the skin and $L$ is the length of the pore. The shape of the pore created by MNs is related to the MNs’ geometry which should be done in the modelling section. To optimize the MNs enhanced diffusion in the system, the crucial factor is the arrangement of MNs on the patch. Based on the imported MNs model, there is function ($g$) introduced to characterize the pores on a square patch which is shown in equation (2.19) (Al-Qallaf and Das, 2009).

\[ g = \frac{n^2R^2}{A} \]  

(2.19)

Where $n$ is the total numbers of MNs per row on the patch, $R$ is the radius of the MNs and $A$ is the area of the patch. The three parameters in the equation (2.19) are used to decide the optimum arrangement of MNs on a certain patch that can achieve highest diffusion rate. However, there are restrictive conditions that keeping the numbers of MNs on the patch within a rational range to avoid overflow (equation 2.20) (Al-Qallaf and Das, 2009).

\[ n_{\text{min}} \leq n \leq n_{\text{max}}; R_{\text{min}} \leq R \leq R_{\text{max}}; A_{\text{min}} \leq A \leq A_{\text{max}} \]  

(2.20)

Another important restrictive condition is the pitch $P_t$ which is defined as the center to center distance between two adjacent MNs. It can ensure the MNs on the patch doesn’t overlap with others and avoid disordered pattern (Al-Qallaf and Das, 2009).

\[ P_t = \frac{\sqrt{A}}{n} \geq \alpha R \]  

(2.21)

Where $\alpha$ is the aspect ratio of the pitch and must be larger than 2 to avoid overlap. Equation (2.21) also indicates the MNs on patch are arranged in square pattern. To achieve other patterns such as triangular, diamond or rectangular, the pitch component in transverse
direction ($P_{in}$) and longitudinal direction ($P_{lm}$) must be specified (Al-Qallaf and Das, 2009). An illustration on diamond pattern is shown in Figure 2.28.

Figure 2.28 A diamond pattern on the MNs patch achieved by adding condition: $P_{in}=P_{lm}=0.707P_t$ ($\theta_t=45^\circ$) (Al-Qallaf and Das, 2009).

After the pattern has chosen, the optimized $\alpha$ will be deduced so the relationship between the pitch and the parameters in equation (2.19) can be found. By applying Fick’s law, the correlation between the permeability and diffusion coefficient of different drug molecules based on those optimum parameters can be calculated which are shown in Table 2.3 (Al-Qallaf and Das, 2009).

Table 2.3 The correlation between different patterns of MNs patch and the permeability of drug solutions. The diffusion coefficient $D$ is related to different drug molecules (Al-Qallaf and Das, 2009).

<table>
<thead>
<tr>
<th>Types pattern</th>
<th>Permeability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Square</td>
<td>$k = 1.6185 \times D - 0.0008$</td>
</tr>
<tr>
<td>Diamond</td>
<td>$k = 0.8125 \times D - 0.0029$</td>
</tr>
<tr>
<td>Triangular</td>
<td>$k = 0.936 \times D + 0.0007$</td>
</tr>
<tr>
<td>Rectangular</td>
<td>$k = 1.622 \times D + 0.0002$</td>
</tr>
</tbody>
</table>

2.2.3.3.2 The simulation of hollow microneedles

The hollow MNs has a channel that can continuously delivery drug solution into the skin. However, the annular wall of the MNs limits the contact area of the drug solution to the skin,
hence the parameter $f$ in the equation 2.18 needs to be redefined which is shown in equation 2.22 (Al-Qallaf and Das, 2009).

$$f = n\pi \frac{(R + W)^2 - R^2}{A}$$  \hspace{1cm} (2.22)

Where $W$ is the annular gap width of the hollow MNs. The drug delivery mechanism of hollow MNs is more complicated comparing to the solid MNs because the drug delivery rate of hollow MNs is not only relying on the diffusion but also depending on the injection process. Therefore, a new concept ‘moving interface’ is introduced to describe the boundary that separating saturated and unsaturated skin tissues. The velocity of the moving interface should be higher during injection than passive diffusion rate which is presented in equation 2.23 (Lv et al., 2006).

$$u_{\text{int}} = \frac{u_0 \varepsilon}{\varepsilon + \alpha (1 - \varepsilon)} e^{- \frac{\beta t}{\varepsilon + \alpha (1 - \varepsilon)}}$$  \hspace{1cm} (2.23)

Where $u_{\text{int}}$ is the velocity of the moving interface, $u_0$ is initial injection velocity, $\alpha$ is the drug solution absorption rate per unit volume tissue, $\beta$ is the absorption coefficient of drug taken by blood stream and $\varepsilon$ is the average porosity of the skin. The results also indicate that higher diffusion rate can be achieved by increasing $u_0$ while the porosity and absorption rate of skin are considered as intrinsic invariables. In the said study the moving interface position is measured in an assumed thickness $x_0=15 \, \mu m$ skin sample which is shown in Figure 2.29.

![Figure 2.29 Influence of injection velocity on the position of the moving interface ($\varepsilon=0.2$, $\alpha=0.01m^3 \text{ liquid}/m^3 \text{ tissue}$, $\beta = 0.005m^3 \text{ liquid}/m^3 \text{ tissue}/s$)) (Lv et al., 2006).](image_url)
2.2.3.3.3 The simulation of dissolving microneedles

The dissolving MNs has similar mechanism to solid MNs when using poke with patch method. However, the drug matrix in the reservoir of dissolving MNs is formulated so it can be released over time. Therefore, the actual amount of drug solution is a time depended function that related to the dissolving rate of the drug matrix, i.e., the volume change rate of a conical dissolving MNs is shown in equation 2.24 (Kim et al., 2015).

\[
\frac{dv_c}{dt} = -\pi \frac{k_D}{\rho}\left(\frac{\tan \theta}{\cos \theta}\right) h^2 \left[c_s - \left(\frac{1 - \beta}{\beta}\right)C\right]
\]

Where \(v_c\) is the volume of the dissolving MNs, \(k_D\) is dissolution rate constant of the drug matrix, \(\rho\) is the density of the drug matrix, \(h\) is the height of the MNs, \(c_s\) is the solubility of the drug matrix in water, \(C\) is the drug concentration in the skin, \(\beta\) is the mass fraction of drug in the MNs and \(\tan \theta\) is the original ratio of radius over height of the MNs. The equation (2.24) indicates the boundary conditions of the simulation are changing with time and highly related to the dissolution rate of the drug formula. Besides, the alignment of the MNs and mass fraction of the drug in the MNs are also adjustable parameters that can affect the diffusion rate.

There are other factors analyzed to improve the accuracy of the simulation according to the viscoelastic properties of the skin, i.e., the force required for full penetration of MNs into the skin (equation 2.25) (Olatunji et al., 2013).

\[
F_{\text{insertion}} = F_{\text{bending}} + F_{\text{indentation}} + F_{\text{cutting}} + F_{\text{buckling}} + F_{\text{friction}}
\]

Where \(F_{\text{bending}}\) is the force that can bend the skin, \(F_{\text{indentation}}\) is the force that the MNs starts disrupting the SC layer, \(F_{\text{cutting}}\) is the force that the MNs starts piercing into the skin, \(F_{\text{buckling}}\) is the force causing skin deformation and \(F_{\text{ friction}}\) is the frictional force during the MNs penetration. The results of However, those force components are not only depending on the mechanical properties of the skin but also affected by the geometry and alignment of the MNs on the patch. The deformation of the skin caused by those forces can be different from the shape of the MNs, thereby changing the results of the simulation.

Moreover, new concepts are also emerging based on the previous parameters to increase the accuracy of modelling and simulation, i.e., effective thickness is introduced which is
calculated from the effective permeability of viable epidermis using Fick’s law (Al-Qallaf et al., 2009). It considers the skin deformation after the MNs insertion in order to give a more realistic value to skin thickness because the MNs reduce the distance between the drug loads to the blood stream. The effective thickness $H_{\text{eff}}$ can be calculated using equation (2.26) (Davidson et al., 2008).

$$H_{\text{eff}} = \frac{D_{\text{VS}} C_{\text{m}}}{J_{\text{ss}}}$$  \hspace{1cm} (2.26)

Where $D_{\text{VS}}$ is the diffusion coefficient in the viable epidermis, $C_{\text{m}}$ is the concentration coated on the MNs.

To apply all these parameters for simulation, the MNs design needs to be digitalized for computer program to process. Finite element method is chosen as the algorithm by most of the studies because it can provide detailed diffusion or force distribution profiles (Shuhu et al., 2013) and the time range allow to apply transient model or steady state (Zhang et al., 2010). The MNs based simulation is the only way to test the efficiency and feasibility of the MNs design apart from the experimental method. The advantages of the simulation is that it can predict the diffusion results before the experiment. The accuracy of the diffusion results relies on the parameters defined at the modelling stage so the theoretical data of those parameters are crucial and if they are well measured, the prediction will be more convincing comparing to the experimental results.

2.3 Summary

The TDD technologies are important drug delivery methods and they are developing fast as reviewed and forecasted in this chapter. The idea of ultrasound and MNs combination is relatively new but shows a promising future. It is expected that the forms and design of MNs patches will be more diverse in the future. Given that portable ultrasound instrument is a common medical device in hospitals, a patient friendly design of the combination are likely to be achieved at a reasonable cost when this concept is matured enough. In order to discuss the scope of combined MNs and ultrasound research, this review chapter has introduced the relevant mechanisms of MNs and sonophoresis as well as pointed out the weak areas in the researches of these two technologies. By working on those weak areas, more combinational approaches can be developed, particularly when the ultrasound and MNs are more developed as methods. Although the combination of MNs and ultrasound seems to make TDD more complicated, it is necessary to achieve both higher and controllable drug delivery
rate. In conclusion, we must state that the MNs and ultrasound combination has a promising future to solve some of the current problems in TDD.

The MNs modelling is another important subject in the MNs research. Although the MNs modelling is still at its very early stage, but its importance needs to be recognized for its great potential. Once the theoretical basis is formed, the MNs modelling will be the most efficient way for both MNs researches and manufactures. With more information acquired from MNs experiment, the interaction between the MNs and the skin as well as the drug diffusion profile in the skin will be unravelled. At that time, MNs models will be massively replacing the experimental works and greatly improve the efficiency of MNs manufactures.
Chapter 3
Proof of concept: permeability enhancement for transdermal delivery of drug molecules using low frequency sonophoresis combined with microneedles

Overview

In this chapter, we use porcine ear skin to simulate human skin and treat the skin samples with both ultrasound and MNs. Bovine serum albumin (BSA) is used as a model of larger molecular weight molecule. Our results show that the permeability of BSA is increased to 1 µm/s with the combination of 1400 µm MNs patch and 15 W ultrasound output which is about ten times higher than the permeability obtained in passive diffusion. Diffusion with only MNs or ultrasound pre-treatment is also tested. The maximum permeability from MNs and ultrasound treatment reached 0.43 µm/s and 0.4 µm/s, respectively. We also performed our experiment to explore the enhancement of transdermal drug delivery of a semi-interpenetrating polymer network (sIPN) formulation. Varying ratios of carboxymethylcellulose and gelatine encapsulating lidocaine was formulated for permeation study. In vitro studies using Franz diffusion cells were used to measure permeation through porcine skin after pre-treatment with a stainless steel MNs array and 20 kHz sonophoresis for 10 minutes. The results revealed significant increases in permeability when both pre-treatments were combined, up to 4.4-fold decrease in the time required to reach therapeutic levels of lidocaine was observed. Sonophoresis only pre-treatment showed minor initial enhancement with a significant enhancement post 2 hours for the vacuum dried formulation and post 1 hour for the rotary dried formulation. MNs application increased initial permeability up to 17-fold with an average up to 4-fold increase.

3.1 Background

The administration of most biotherapeutics and vaccines e.g. lidocaine, are traditionally done via hypodermic needles as a low cost, fast acting and effective method of drug delivery (Kim et al., 2012; Hedge et al., 2011). However there are numerous rationales for the pursuit of alternative drug administration methods backed by the European paediatric drug legislation to develop an "easy to administer" and minimally invasive drug delivery method (Shah et al., 2011). Such rationales include the need to increase safety amongst patients and healthcare
providers, increased compliance with those whom possess a fear of needles, reduced discomfort and pain especially in the case of anaesthetics as well as improved ease of delivery (Gill and Pausnitz, 2007; Giudice and Campbell, 2006; Li et al., 2010). Oral administration can overcome many of the disadvantages associated with direct injection of drugs. Constraints in oral delivery are due to a low bioavailability of lidocaine with a mean of 32 to 35 % caused by hepatic enzymes thus limiting the number of drugs able to be effectively delivered orally (Shipton, 2012; Benet et al., 1996; De Boer et al., 1979; Huet and Lelorier, 1980). Similarly transdermal drug delivery (TDD) is an alternative administration method in overcoming barriers associated with direct injection and oral administration of drugs (Polat et al., 2011). Until the 1940s, TDD method was considered as one of the most essential methods for drug delivery through the parenteral drug delivery routes (Hillery et al., 2001). At that time, the dosage forms of TDD only had topical creams and ointments, and they relied on passive diffusion of the drug molecules through skin. The physicochemical properties of the drugs were generally hydrophobic in nature because of the lipophilicity of skin (Schaefer H, 1996) and, since the hydrophilic drug formulations seemed to have more tendencies to vapourise than to permeate through the skin. The TDD methods can avoid the gastrointestinal and liver metabolisms which may be severe for the delivery of some proteins and vaccines. As the scope of TDD has increased over the decades, it can be considered as a main alternative to other parental drug delivery routes, e.g., oral delivery route (Hadgraft and Guy, 1989). With the continued development of pharmaceutical and related sciences, more drugs are being invented which may need to be delivered transdermally. The molecular weight of these drugs can vary from less than 100 Da to over 150 kDa. Several technologies have been developed to increase the permeability of these drugs in skin. But, in the delivery of large molecules such as insulin, bovine serum albumin (BSA) or tetanus toxoid, the delivery rates of the molecule are still found to be either low or undetectable according to many literatures (Dahlan et al., 2009; Tachibana, 1992; Devin et al., 2003; Dahlan et al., 2009). Due to the desired pain free nature of the transdermal drug delivery technologies (Barry, 2001), the permeability increment of large molecules that just relied on one delivery method, such as passive diffusion, can be difficult. As such, researches on combining individual technology are necessary so as to multiply their advantages in enhancing the drug permeability.

In addressing these issues, we explore a way to combine ultrasound generated cavitation with MNs patch in the current work for delivering large molecules, both of which are chosen from well accepted TDD methods. As well known, the TDD methods can be divided into three categories in terms of their mechanisms, namely, (1) diffusion of small ions or charged molecules under an electrical field known as iontophoresis and electroporation (Banga and
Prausnitz, 1998; Banga et al., 1999); (2) increase of drug solubility in the donor solution or cooperate with chemical enhancers to increase permeability (Johnson et al., 1996; Barry, 1987); (3) penetrate or rub the skin surface to change the structure of the skin including sonophoresis and MNs (Olatunji et al., 2012; Mitragotri and Kost, 2004). Both ultrasound and MNs create pores/holes of different scales in skin. The holes created by MNs are generally visible through naked eyes and do not close up immediately. On the other hand, the ultrasound is focused on fluctuating the size of naturally occurring skin pores by creating cavitation in the skin. At the moment, there are very few reports on sonophoresis enhanced delivery of large molecules because the pores created by ultrasound are often not sufficient to deliver those molecules.

The performance of the MNs patch is also limited by its own properties, e.g., the length of the needles, the needle density in the patch, the geometry of each needle, and, materials of needles (Al-Qallaf and Das, 2009; Nayak and Das, 2013). The MNs not only create holes on the surface of the skin but also change the skin property (e.g., effective viscoelasticity and transport properties) of the affected area (Olatunji et al., 2012; Olatunji O et al., 2013). It will leave a good basis after the treatment so that the ultrasound generated cavitation can become more efficient on that field. The ultrasound and MNs can be then combined as an effective group. However, the other two technologies, namely, chemical enhancers and electrical fields, do not seem to be readily suitable for delivery of large molecules because the chemical enhancers are most efficient in the transportation of small molecules and the electrical fields can only deliver ionic compounds (Michael, 2002).

The focus of this chapter is to carry out pre-treatment of skin by MNs so as to alter the skin property, and then apply ultrasound field on the pre-treated area to let the cavitation further increase the permeability of the skin. As model molecules for the permeation measurement bovine serum albumin (BSA) and lidocaine are chosen in this study. The ideal anaesthetic can be described as one that provides rapid, prolonged and effective localised anaesthesia via a non-intimidating mechanism that induces no pain and causes no adverse local tissue reaction (Zhang et al., 2012). Lidocaine has been noted as the most versatile and widely used local anaesthetic, commonly administered via intradermal injection and is known to cause significant pain. Alternatives such as ELMA, a topical form to administer lidocaine, require at least an hour of application to achieve effective analgesia therefore limiting its use especially with emergency situations (Tachibana and Tachibana, 1993). Iontophoresis has been used to effectively deliver therapeutic levels of lidocaine however patients have stressed a discomfort caused by tingling or itching sensations due to the electrodes, therefore not a viable method (Rose et al., 2002). A sIPN formulation was chosen due to its
flexible properties and ability to encapsulate considerable amounts of fluids, lidocaine in this instance (Aalaie et al., 2013). BSA is a common protein derived from cows and has a molecular weight over 60,000 Da. The reason of choosing BSA for the experiments is because BSA is a hydrophilic molecule and its molecular size ranges between the sizes of small peptide molecules and large vaccines. Also, it does not metabolise in skin. Due to its molecular cut-off, BSA should not diffuse passively through the SC layer. However, if the skin is treated with other factors such as MNs, the molecule may pass through. To help BSA and lidocaine NaCMC/gel hydrogel passing through the SC layer of the skin, the sonophoresis (or phonophoresis) combined with MNs patch method is employed as discussed further below.

As well known, sonophoresis uses ultrasound to enhance the permeability of the skin. The two main adjustable parameters of ultrasound are the intensity ‘I’ (power on unit area) and frequency ‘f’ (number of cycles per second) (Frederick and Kremkau, 1983). These two parameters work in a synergetic manner. As Tezel et al. reported, there is threshold intensity for every different frequency. For example, the threshold intensity is 0.11 W/cm² at 19.6 kHz and greater than 2 W/cm² at 93.4 kHz (Tezel et al., 2001). Once the intensity exceeds its threshold, the enhancement of drug delivery increases significantly as well. Although the experiment of Tezel et al. was based on the measurement of porcine skin conductivity, it gives an idea that in the delivery of a specific drug, ultrasound output parameters can be optimised by careful selection of an intensity range for a specific frequency. For example, at a frequency of 1 MHz, the permeability increase for delivery of mannitol (182 Da) through rat’s skin is not detectable at a frequency of 0.1 W/cm² in comparison to passive diffusion of the drug. However, it increases by 2 folds at an intensity of 1.5 W/cm² and 4 folds at an intensity of 2 W/cm² (Macheta et al., 1998; Meidan et al., 1998). Besides the intensity and frequency of ultrasound, the scale of the permeability enhancement for a specific molecule is also strongly connected to its molecular size. For cortisol (382 Da) under the same conditions (1 MHz, 1.5 W/cm²), the increment of permeability is less than 20% (Macheta et al., 1998). The low frequency sonophoresis (LFS) is defined to be within the range of 20–100 kHz and the high frequency sonophoresis (HFS) is usually for above 0.7 MHz (Polat et al., 2011). The mechanism by which enhanced permeability is achieved via ultrasound can be linked to a number of physical phenomena including thermal effects, formation of cavitation, mechanical effects and convective localised fluid velocities in skin (Lavon and Kost, 2004). However, in the ultrasound pre-treatment experiment, it is generally accepted that inertial cavitation is the largest contributor to the enhancement in skin permeability. Boucaud et al. (2002) report that the blood level of in vivo rat drops to half when one applies insulin (5.8 kDa) with ultrasound (2.5 W/cm², 20 kHz, 15 min). Besides, the duty cycles, treatment time
and, the distance between ultrasound transducer and target also need to be considered carefully for specific ultrasonic application.

As mentioned previously, the primary mechanism in sonophoresis application is cavitation effect, which have been discussed in length by many authors (Mitragotri and Kost, 2004; Polat et al., 2011). When the ultrasound waves compress and tense a liquid, the liquid pressure falls below its vapour pressure which forms the cavitation. The cavitation is divided into two types, namely, stable and transient cavitations (or inertial cavitation), which are discriminated by how long the bubbles survive (Polat et al., 2011). Inertial cavitation occurs due to pressure variations induced by ultrasound, resulting in rapid growth and collapse of bubbles formed in the coupling medium. The collapsing of the aforementioned bubbles near skin surface will cause micro-jets due to asymmetrically release of energy. These micro-jets have been confirmed as the main contributors to the permeability increment (Wolloch and Kost, 2010). The effects of ultrasound have been studied for the enhancement of transdermal lidocaine administration delivery with significant enhancement demonstrated results for with both pulsed and continuous output mode of LFS (Ebrahimi et al., 2012). The cavitation generated during high frequency ultrasound treatment is much smaller in size compared to those for low frequency condition because of the relationship between the frequency and the bubble radius, i.e. $C = f \times r$, where $C$ is a constant determined by the properties of the solution, $f$ is the frequency of the ultrasound and $r$ is the radius of the bubble (Gaertner, 1954). For example, bubbles generated in water caused by 20 kHz ultrasound is typically 150 µm in radius, but it is only 1 µm when the ultrasound frequency is increased up to 3 MHz (Leighton, 1998). The larger bubbles can cause more disruption when they burst. For this purpose, 20 kHz is chosen in most sonophoresis TDD experiments (Polat et al., 2011).

There are other factors that may affect the skin permeability. The most obvious phenomenon during the ultrasound application is the temperature rise of the skin. The skin absorbs the mechanical energy of sound field. This increases the temperature which may be significant when the ultrasound frequency and intensity go up. This means that the energy would be stored in skin rather than transmit through (Bommannan et al., 1992) affecting the skin permeability. The parameters of ultrasound inputs must be kept to a safe range because high temperature can cause skin injury. It has been reported that when the temperature reaches 43°C or higher and stays at that level for 60 minutes or longer, it can restrain cellular reproduction. If the temperature increases to 56°C, it can cause irreversible cell death and necrosis (Kennedy et al., 2003). This is the main reason why we choose to apply ultrasound prior to the diffusion experiment rather than applying it simultaneously.
MNs patch is a kind of technology which sits at the interface between transdermal patches and hypodermic needles, attempting to gain the advantages and eliminate the disadvantages of each (Al-Qallaf and Das, 2009; Al-Qallaf et al., 2009). In vitro research has shown permeability enhancement of two to four orders of magnitude (Van Der Maaden et al., 2012). There are many reports on the delivery of large molecules using MNs patch. Martanto et al (Martanto et al., 2004) reported the delivery of insulin through rat’s skin using 1000 µm length MNs patch. They used the MNs patch to repeatedly pierce the same site and as a result the insulin blood level dropped to one quarter. Dissolving MNs also have been used for delivering large molecules like lysozyme (14 kDa) or BSA (60 kDa) but the drug loading on the MNs patch is typically less than 1–2 mg which seem to limit their application (Haripriya and Ajay, 2011).

This study will focus on solid MNs utilising the ‘poke and patch’ technique, the main advantages being the technical simplicity required for synthesis therefore cost reduction. The other major advantage being that an extended release is possible. However in comparison to the other forms of MNs, solid MNs tend to have lower drug delivery rates and the process is two stage rather than a one stage technique (Van Der Maaden et al., 2012). In our study, we use solid MNs because it is more rigid in structure in comparison to the hollow MNs. The MNs can create a porous basis as well as dents and other structural changes on the skin for diffusion rate to increase. It weakens the resistant functionality of the SC layer and exposes parts of the underneath epidermis to the molecule of interest. The ultrasound treatment is then applied on the MNs pre-treated area to further enhance the skin permeability. The output of ultrasound uses continuous wave mode to generate more bubbles in the limited time duration. These bubbles will be in contact with both SC layer and MNs pre-treated area so the permeability is significantly increased. The whole permeation study is based on a Franz diffusion cells system so the amount of the BSA or lidocaine NaCMC/gel hydrogel passes through the skin can be quantified. Further, the potential of MNs for delivery of lidocaine via a sIPN formulation has previously been reviewed with the conclusion there is a gap for lidocaine MNs products (Nayak and Das, 2013). To try and exploit this potential the main aim of the study is to use a MNs array and low frequency sonophoresis technology together as a pre-treatment to meet the definition of an ideal anaesthetic method and a reliable method that can deliver large molecules efficiently.
3.2 Materials and methods

3.2.1 Materials

Bovine serum albumin (BSA) and methylene blue were obtained from Sigma-Albrich (Gillingham, Dorset, UK). Trifluoroacetic acid (TFA) and acetonitrile were bought from Fisher Scientific (Loughborough, UK) for using them as the high-performance liquid chromatography (HPLC) mobile phases. All deionized water for use in permeation experiment was purified using Milli-Q System (Billerica, MA, USA).

Sodium carboxymethylcellulose (0.9 D.S.; 250kD), sorbitan mono-oleate (SPAN 80), glutaraldehyde 50% w/w, paraffin liquid (0.827-0.89 g/ml), lidocaine hydrochloride (288.81g/mol), porcine gelatine (Type A) were purchased from Sigma Aldrich Ltd, Dorset, UK. Acetic acid, ammonium bicarbonate 99+ wt % and n-hexane (95% w/w) were purchased from Fisher Scientific UK, Leicestershire, UK.

Figure 3.1 The porcine ear is sliced into four sections. The thickness of these sections increases from section 1 to 4.
Porcine ear skins were purchased from a local abattoir. The porcine ears were collected from 5-6 months old piglets. The skin samples were then kept in a cool-box for the transport from the abattoir to our laboratory. The ears were cut into four sections as shown in Figure 3.1. Section 1 is too thin and it seems to get damaged during the process of separating the skin from the cartilage in the ears. Therefore, section 1 was not used in our experiments. Section 4 was also rejected because of the existence of excess underneath fats and muscles. These tissues are likely to affect the thickness of the skin samples varying in a wide range and will probably cause inaccuracies during the permeation results.

Sections 2 and 3 were cut into small pieces and wrapped in foil papers and then flash frozen in a tank filled with liquid nitrogen. According to the skin graft preservation procedure (Marcia, 2011), the skin samples were dipped in the liquid nitrogen until it stopped boiling (about 40 s) which indicates that the temperature of the skin has reached -196°C. The skin samples are then kept into a container in the freezer that is set to -22°C. Before the permeation experiments, these samples are allowed to stay in room temperature for 2 hours until which they are fully thawed. A surgical scalpel is used to separate full thickness porcine ear skin from the underneath cartilage by carefully cutting off the connective tissues in between. To assure the integrity of the skin samples, some of the connective tissues under the skin is removed carefully from the cartilage to avoid irrelevant tissues.

### 3.2.2 Preparation of lidocaine formulation

Paraffin oil (100 ml) was stirred continuously up to 400rpm (Eurostar, UK). Span 80 (0.5 %wt) was dispersed in ambient conditions. To this NaCMC (1.24 %wt) in ultrapure water was added drop wise, and depending on the polymeric ratio, varying amounts of gelatine (Table 3.1) in ultrapure water at 35-40°C. Following this the pH of the solution was reduced to pH 4 via the addition of acetic acid (~ 3 % wt). Lidocaine HCl (2.44 %wt) in ultrapure water at 25°C was added to the polymer mixture. The mixture was then cooled to 5-10°C for 30 minutes to initially harden the suspension. Glutaraldehyde (0.11 % wt) was added to the emulsion as a cross linker. Upon returning to room temperature the suspension mixture was stirred for 2 hours at approximately 1000rpm to ensure thorough mixing. The suspension formulation was then left to stand until a distinct 2 layer boundary was observed after which the formulation was left overnight at 1-5°C. Excess Paraffin liquid was removed by first mixing with n-hexane (50 % v/v), top organic layer was syringed before placing the suspension formulation in a vacuum oven at a pressure of 160mmHg, temperature of 25°C for 8 hours. In the case of F5 residual paraffin and n-hexane were removed by rotary evaporation (Heidolph UK). Following this the formulation was washed with DI water and
filtered (Whatman) for removal of unbound lidocaine before further characterisation. The formulation intents to extend the drug release time in the skin while encapsulates sufficient amount of lidocaine drug solution. The characterizations of the formulation such as: zeta potentials, viscoelastic properties, controlled release time of the formulation can be found in details in our previous group paper (Nayak et al., 2014).

Table 3.1: Various ratios and components of formulations synthesised as well as mean particle diameter size.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>NaCMC (g% w/v)</th>
<th>Gelatine (g% w/v)</th>
<th>Lidocaine (g% w/v)</th>
<th>NaCMC:Gelatine ratio</th>
<th>Drier Type</th>
<th>Mean Particle Diameter ± S.D. (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>1.25</td>
<td>2.00</td>
<td>2.50</td>
<td>1:1.6</td>
<td>Vacuum</td>
<td>5.89 ± 0.0026</td>
</tr>
<tr>
<td>F2</td>
<td>1.25</td>
<td>2.50</td>
<td>2.50</td>
<td>1:2.00</td>
<td>Vacuum</td>
<td>6.04 ± 0.0027</td>
</tr>
<tr>
<td>F3</td>
<td>1.25</td>
<td>2.91</td>
<td>2.50</td>
<td>1:2.33</td>
<td>Vacuum</td>
<td>6.81 ± 0.0029</td>
</tr>
<tr>
<td>F4</td>
<td>1.25</td>
<td>3.33</td>
<td>2.50</td>
<td>1:2.67</td>
<td>Vacuum</td>
<td>7.42 ± 0.0029</td>
</tr>
<tr>
<td>F5</td>
<td>1.25</td>
<td>3.33</td>
<td>2.50</td>
<td>1:2.67</td>
<td>Rotary</td>
<td>14.60 ± 0.0067</td>
</tr>
</tbody>
</table>

3.2.3 Treatment on skin with ultrasound and microneedles

A commercially assembled ultrasound system (Branson Digital Sonifier 450 Danbury, CT, USA), which includes an ultrasound generator and an ultrasound transducer, is used to treat the skin in this work. The frequency of the ultrasound is fixed at 20 kHz while the input powers are varied between 4 and 400 W. The transducer is mounted on a specially designed ultrasound holder to ensure the distance between the tip of the ultrasound transducer and the skin sample is fixed at 1mm. The coupling medium for ultrasound transmission during the pretreatment for all permeation studies is deionized water. The setup of the ultrasound system is shown in Figure 3.2.
To ensure that the sound field indeed affects the localised transport regions (LTRs) (Tachibana, 1992), the transducer is set to 12 W and kept approximately 1 mm away from the skin surface. The ultrasound is then applied to the skin for 10 min and its effect is visualised as follows. The skin sample is completely merged in 100 ml water and adhered to the bottom of the beaker. One drop of methylene blue is then dissolved in the water which is distributed immediately by the ultrasound transducer. Due to the LTRs effect, the sound field will not spread on the skin consistently. However, as the skin is merged in the methylene blue solution, the entire skin surface turns into blue. But the area that is most affected by the sound field shows deeper blue than the surrounding untreated area which indicates that more dye molecules has permeated into the regions through the skin area that are affected most by the sound field. This is shown in Figure 3.3. In terms of choosing the most affected regions for permeation study, the centre of the skin sample is employed.
Figure 3.3 The identification for the ultrasound field, the deep blue area shows the ultrasound generated cavitation affected area.

Two commercial MN patches, namely, AdminPatch® Array 1200 MN (1100 µm length) and AdminPatch® Array 1500 MN array (1400 µm length) (AdminMed, Sunnyvale, California, USA) are employed to penetrate the skin sample with 43 and 31 individual micro-needles on each patch, respectively. The 1400 µm MNs patch is shown in Figure 3.4.

Figure 3.4 The 1400 µm length MNs patch from AdminPatch.

In order to identify the hole size created by the MNs, a staining experiment is conducted which allows visualization of the holes and confirm that the MNs have pierced the skin. For this purpose, the methylene blue solution is diluted to 10% v/v. The skin sample is treated with the MNs patch for 5 min then merged in the dye solution for 1 min. The dye solution is
then washed off with deionized water and the skin sample is observed under the microscope. Figure 3.5 shows that some typical holes size which becomes smaller after 2 hours.

![Microneedles treated holes](image)

Figure 3.5 The left picture shows the MNs treated hole immediately after staining; the right picture shows the size of the same hole after 2 hours. The scales in both pictures are 300 µm.

### 3.2.4 Diffusion cells

A Franz diffusion cell, which is commonly used for measuring drug permeability in skin, was used in this work to determine the permeability. It is consisted of four parts: the water tank, the heater (VTC 200 Logan Instrument Corporation, NJ, USA), the magnetic plate (FDC-6 Logan Instrument Corporation, NJ, USA) and the diffusion cells (Figure 3.6). The water in the water tank is pumped through the whole system. In order to simulate body temperature, the water is heated to 37°C which warms all the diffusion cells by going through their jacketed compartments. The real temperature may be different by a margin of ±1 degree centigrade from the set value due to the inaccuracies in the sensor.

For the individual cells (Figure 3.7), there are two compartments which are the main parts of the cell: the donor compartment on the top and the receiving compartment at the bottom. The skin sample sits in between of the two compartments and it is fixed using a clipper. A magnetic stirrer is used to mix the receiving solution which represents the blood circulation beneath the skin.

For conducting the permeability measurements, the donor compartment is filled with BSA solution of a certain concentration while the receiving compartment is filled with deionized water. The liquid surface level is different between donor compartment and the receiving compartment so that a pressure head exists. This pressure head can generate convection...
effect which may affect the permeability. To balance the pressure difference, a parafilm is used to seal the donor compartment to keep pressure at $P_2 = P_1 - \rho gH$, where $P_1$ is equal to standard atmosphere, $P_2$ is the pressure under the parafilm, $\rho$ is the density of the BSA solution, $g$ is acceleration due to gravity and $H$ is the pressure head. However, the pressure head in the delivery of Lidocaine NaCMC/gel hydrogel can be neglected due to the high viscosity and less quantity (0.1±0.03g) of the suspension. Therefore, the donor compartment is not sealed by parafilm during the diffusion experiment for the delivery of lidocaine.

Figure 3.6 the whole setup of Franz diffusion cell system: A) Magnetic plate; B) Water tank; C) Heater; D) Diffusion cells; E) Pump. The circulation from water tank goes to the heater, then warms all cells and finally goes back to the water tank.
Figure 3.7 The individual FDC cell, the donor compartment is on the top, the receiving compartment is on the bottom and the skin sample is in between of them. A water circulation is used to keep the receiving compartment at 37°C.

The high performance liquid chromatography (HPLC) is used to analyse the BSA concentration of the sample taken from the receiving compartment. The samples that kept in 5 ml vials is mixed with 95% of mobile phase A (0.1% v/v TFA in H2O) and 5% mobile phase B (0.08% v/v TFA in acetonitrile) and pumped through the HPLC column at a rate of 10 µl/min. The system will be purged for 20 min with pure mobile phase before the experiment and a degasser is used to eliminate any bubbles remain in the mobile phase before it enters the column. An ultraviolet (UV) detector is set at the end of the column to record light absorbance unit under 232 nm wavelength. A calibration curve is made beforehand using BSA solution with known concentration compares to the recorded light absorbance unit in order to identify the relationship between the light absorbance and the concentration of BSA. The calibration curve is highly linear and can be found in the appendix. The skin samples are also soaked in the water for 2 hrs and tested using the aforementioned wavelength to make sure the contaminants from the skin will not interfere the accuracy of BSA concentration. It also indicates the retention time of BSA curve which is at 13 min in the run. To calculate the permeability of the skin, the Fick’s law is used:
\[ J_{ss} = \frac{Q}{AT} = \frac{D \Delta C_v}{h} \]  

(3.1)

Where \( J_{ss} \) is the total flux, \( Q \) is the total mass of BSA in the receiving compartment, \( A \) is the affected diffusion area which is fixed to 1.33 cm\(^2\), \( T \) is the time interval between each sample, \( \Delta C_v \) is the concentration difference between the donor and receiving compartment, \( D \) is the diffusion coefficient of the skin. The volume of the receiving compartment is 5 ml in this case.

Using the concentration data acquired from HPLC, the total amount of BSA in the receiving compartment \( Q \) can be calculated. After calculating the total flux, the permeability of the skin \( k_p \) is deduced from the following equation:

\[ k_p = \frac{J_{ss}}{\Delta C_v} \]  

(3.2)

Where the \( \Delta C_v \) is calculated just using the concentration in the donor compartment because the concentration of BSA in the receiving compartment is negligible compared to the concentration in donor compartment.

All the diffusion experiment and temperature measurement data are presented as arithmetic mean values ± standard deviation (SD). For each set of experiment (passive, MNs and ultrasound), six individual skin samples are used to calculate the mean values. The statistical data are presented directly in the figures.

### 3.2.5 Histological study

The determination of MNs insertion depth into skin by post MNs treatment of skin was adapted from Cheung et al (2014). First, the skin sample is pretreated using 1100 µm MNs patch for 5 min. Then, the porcine skin sample is stained using methylene blue (50% v/v) and merged into embedding compound (Bright Cryo-m-Bed, Huntingdon, UK) which is filled in a cuboid mould. The whole sample is then put inside the microtome (Bright Cryostat 5030, Huntingdon, UK) to solidify. The frozen sample is cut into 15 µm slices and analysed under the microscope for the histology.
3.3 Results and discussions

3.3.1 Staining experiment

As mentioned before, a series of staining experiments has been performed on typical skin samples to confirm that the MNs create holes in the skin under normal thumb pressure (~10N). The porcine skins that were bought from the local abattoir were de-haired by the butcher. The skin samples seem to provide a clear vision of the hair follicles besides the pores on the skin surface as shown in Figure 3.8. Any micro-pores created by the ultrasound cavitation are undetectable. But a dye solution can reveal the ultrasound effect on the treated area on the skin surface. Furthermore, by magnifying the ultrasound affected area, a qualitative comparison of skin surface before and after the ultrasound treatment can be done under the microscope. For this purpose, two samples are obtained from the same piece of skin. They are immersed in the dye solution with and without ultrasound treatment, respectively. In the former case, the output power of ultrasound is set at 12 W while the distance between the ultrasound horn and the skin surface is set at 1 mm. The skin sample with ultrasound treatment shows higher permeability. The results of the diffusion difference are compared in Figure 3.8.

![Figure 3.8](image)

Figure 3.8 The pictures show the difference on skin surface before and after 10 min ultrasound treatment; The left picture shows the skin sample before treatment. Hair follicles are also marked with black circles.

The staining experiment is also applied on the MNs penetrated skin sample to analyse the effectiveness of the MNs using microtome. The MNs that are employed in the histological experiment are 1100 µm in length. The purpose of the histological experiment is to determine the insertion depth of this MNs patch under thumb pressure for which post-microneedle treated skin is micrograph imaged (Figure 3.9). According to Figure 3.9, the
insertion depth is between 300 µm and 400 µm which are much lower than the real length of MNs. This is caused by several reasons, such as the viscoelastic properties of the skin, the geometry of the MNs and the insertion force. This reduced insertion depth can further affect the permeation results.

![Image](image_url)

Figure 3.9 The MNs insertion depth of skin sample using 1100 µm MNs under thumb pressure. The histological studies shows that although the MNs are 1100 µm, for the MNs density in the array and force applied, they creates holes of approximately 400 µm.

### 3.3.2 Measurement of passive diffusion of BSA

In principle, the skin should block any molecules that are larger than 500 Da (Brown et al., 2006) with a partition coefficient between 1-5 (Kushner, 2006; Kalia and Guy, 2001) passing through its top layer. BSA has molecular weight of 60,000 Da which is much higher than 500 Da and, as such, the BSA permeability should be approximately zero in the passive diffusion experiment. However, due to the fact that there are many imperfections in the skin and the skins are treated by the butcher, some of the BSA molecules still diffuse through the skin. In the skins that were used in these experiments, an average permeability value of approximately 0.11 µm/s has been obtained initially. A BSA solution of 1000 ppm is used as a standard concentration in all permeation experiment. But in order to discriminate the concentration effect in the permeation study, passive diffusion experiments that involve three different concentrations of BSA solution (500 ppm, 1000 ppm and 2000 ppm) are conducted at the beginning. In each permeation study for a certain concentration, six skin samples are used and the results are shown in Figure 3.10. Although the donor concentrations vary significantly, the results show that the permeation values are not affected considerably by
the large variation of BSA concentrations which moves from ~ 0.06 µm/s for 500 ppm to ~ 0.14 µm/s for 2000 ppm.

![Figure 3.10 Passive diffusion with different BSA concentrations of 500 ppm, 1000 ppm and 2000 ppm (results represent mean ± SD values based on data from 6 skin samples).](image)

### 3.3.3 Measurement of passive diffusion of lidocaine NaCMC/gel hydrogel

Passive diffusion permeation experiments were carried out on the lidocaine NaCMC/gel hydrogel in order to provide a control from which any pre-treatment enhancements can be observed. Results (Figure 3.11) demonstrated F1 to be the most effective, this could be due to the combination of higher zeta potential (less flocculation) and smaller particle size contributing to an increased permeation. All lidocaine NaCMC/GEL ratio results have demonstrated a low initial permeation of all formulations- maximum of 0.3µg/ml at t=0.5h. It could be assumed initially lidocaine is diffusing through the fresh skin hence the low initial concentration rates. Due to the requirements of lidocaine as an fast acting anaesthetic the current results confirm enhancement of permeation is required if therapeutic levels of lidocaine (1.5-5µg/ml) are to be reached within a suitable time frame for this technique to be of practical use. From Figure 3.11 it can also be seen that cumulative lidocaine levels tend to stabilise post 4 hours, where equilibrium is reached. F4 & F5 were chosen to be studied for further enhancement via pre-treatment.
3.3.4 Diffusion of BSA with sonophoresis

Sonophoresis can greatly increase permeability for small molecules which has been reported in a number of papers (Meidan et al., 1998; Levy et al., 1989). At 20 kHz, the bubbles generated by ultrasound have their maximum size (Gaertner, 1954) so they can produce the highest damage to the SC layer. But in the delivery of large molecule like BSA, a relatively high output power is essential to create enough cavitation. At lower power range, the permeability is not significantly increased. As Bangtao et al. (2010) have reported, at 20 kHz 0.5 W output power, the BSA permeability is below 0.1 µm/s for 8 hours duration. In Figure 3.12, the results of passive diffusion and ultrasound pre-treatment at different output powers (3W, 6W and 9W) have been shown for a 5 hour experimental duration. In each permeation study, six skin samples are involved and they have been treated with ultrasound at certain power for 5 min. The results indicate that at low output power range the ultrasound enhanced permeability is not remarkable. At 9 W output power, the permeability reaches about 0.26 µm/s while non-ultrasound treated permeability is about 0.11 µm/s. The permeability is doubled but the increment is still not significant.
To optimise the sonophoresis effect, the power of ultrasound must be increased to a higher level and a longer pre-treatment time. In other words, the ultrasound output must reach the threshold which has been mentioned earlier. In order to find this threshold, a permeation study on the different output powers is conducted. In Figure 3.13, the ultrasound output power is varied from 3 to 21 W. Further, the pre-treatment time is varied between 5 min to 20 min. As evident in the figure, at 21 W with 20 min pre-treatment time, the permeability is significantly increased to approximately 3.5 μm/s. However, the skin sample is damaged at a visible level, which means that high temperature and pain will be caused as well if these are applied to a human. To avoid the experiment results go into the decoupling stage where the skin is irreversibly damaged, the power must be chosen so as to maintain the ultrasound treatment at a safe level.
In Figure 3.14, the temperature accompanied with ultrasound pre-treatment is reported. At 10 min treatment time, no significant temperature increase on the skin surface has been recorded. The ultrasound effect is not high enough even in a relatively high power output. At 21 W output, the temperature reaches approximately 33°C which is below the human body temperature. In that case, 10 min treatment with ultrasound can be regarded as safe.

Another issue which needs to be pointed out about the temperature rise is the trend of the temperature curve. If the temperature suddenly rises to a certain level, it may cause uncomfortable experiences to humans. In Figure 3.15, temperature change under 15 W ultrasound treatment for 10 min is recorded every 10 seconds. The results show that the temperature rises smoothly at a constant rate of temperature increase. In the same figure, the temperature decrease of the skin sample is recorded. As evident, compared to the cooling curve, the temperature increment rate is quite slow. In such case, the experiences under the ultrasound treatment should be mild.
Figure 3.14 The temperature changes with different ultrasound power for 10 min, the reference room temperature is 20°C (results represent mean ± SD values based on data from 6 skin samples).

Figure 3.15 Temperature change during and after 15W ultrasound treatment for 10 min.

3.3.5 Diffusion of lidocaine NaCMC/gel hydrogel with sonophoresis

To observe the effect of power and application time of LFS has on the permeation of lidocaine NaCMC/gel hydrogel, LFS was applied continuously with varying power and exposure time as shown in Figure 3.16. Theoretically the exposure of LFS should form cavities in the skin to aid permeation. However lidocaine transport through the skin saw no significant enhancement up to 2 hours after which a significant enhancement (T-test P<0.026) was observed. The results conclude that an increase in power has a greater...
enhancement effect compared to an increase in LFS exposure time however no significant increase in lidocaine transport through the skin was observed during the initial stages of drug application. It is predicted that a higher LFS power level would further increase diffusion however the risk of thermal effects would be too high for this to be of practical use.

Figure 3.16 F4 PD and comparative pre-treatment with ultrasound at 15W and 18W for 5 and 10 minutes respectively.

### 3.3.6 Microneedles enhanced diffusion of BSA

To further increase the permeability, a MNs patch is used. Before combining the ultrasound with MNs patch, a permeation study which is solely based on the MNs is conducted. Devin et al. (Devin et al., 2003) reported that the permeability of BSA using a 150 µm long MNs patch is about 0.02 µm/s. As the permeability increase is nearly undetectable for BSA with smaller MNs (as found in our experiments but not published anywhere), a longer MNs patch is preferred in this study. For the results in Figure 3.17, the MNs patches with 1.4 mm and 1.1 mm lengths are applied to the skin sample for 10 min under a certain pressure of 1 MPa. A 5 hours permeation study is then conducted similar to the passive diffusion experiments.
In comparison with Figure 3.13, the MNs patch increases the permeability to about 0.43 µm/s while the ultrasound at 15W can increases the permeability to about 0.4 µm/s. From Figure 3.13, we find that the best ultrasound output power should be 15 W with 10 min pre-treatment time. Although the 21 W can gives higher permeability, according to its performance at 20 min application time it reaches the decoupling level at this condition and should be avoided in practice. The 15 W output power with 10 min pre-treatment time turns out to be the best parameters for enhancing the permeability and it is also at a safe level. These ultrasound output parameters are then applied on the MNs pre-treated skin to investigate any further permeability enhancement.

![Graph showing permeability over time with different treatment options](image)

Figure 3.17 MNs pre-treatment for 10 min (results represent mean ± SD values based on data from 6 skin samples).

### 3.3.7 Microneedles enhanced diffusion of lidocaine NaCMC/gel hydrogel

To observe the effect varying MNs application time has on the permeation of lidocaine NaCMC/gel hydrogel, MNs were applied for 3 and 5 minutes. Results for MNs only pre-treatment showed a dramatic increase in permeation throughout the experiment for both the 3 & 5 minute patch duration as shown in Figure 3.18. A statistically significant difference (P<0.04) was observed for patch application duration. Initial (t=0.5h) permeation for the 3 & 5 minute patch duration resulted in a 9 and 17 fold increase, respectively. After which an average of a 3 fold increase in permeation was observed for the 3 minute patch duration and a 4 fold increase for the 5 minute patch application. The results indicate that therapeutic
levels of lidocaine could theoretically be reached within 9 minutes of application, in comparison to no pre-treatment requiring 40 minutes.

Figure 3.18 F4 adapting a MNs patch for a 3 minute and 5 minute pre-treatment duration for Lidocaine NaCMC/GEL 1:2.66.

3.3.8 Ultrasound combined with microneedles patch for BSA

In Figure 3.19, different ultrasound powers with a 10 min treatment time are applied on the MNs pre-treated basis. According to the results presented previously, we select the ultrasound powers that have better enhancement effect to combine with the two MNs patches. The results in Figure 3.19 show that the permeability is significantly increased when using the 15 W ultrasound output power and 10 min treatment time combined with the MNs. It also indicates that the thresholds of the synergetic power at 20 kHz are in between of 12W and 18W. Therefore in cooperation with MNs pre-treatment, the permeability reaches a much higher level, which is approximately 1 µm/s.
3.3.9 Ultrasound combined with microneedles patch for lidocaine NaCMC/gel hydrogel

We have also studied the enhancement of ultrasound combined with MNs patch on the diffusion of F4 and F5 lidocaine NaCMC/gel hydrogel. Both pre-treatments were combined and studied for further permeation enhancement in comparison to MNs or LFS pre-treatment only. Combining a 10 minute application of 18W LFS followed by a 5 minute application of MNs saw initial permeation increase 23 fold with an 4.8 fold average increase over 30 minutes of application. Figure 3.20 shows the various combinations of treatments. Therapeutic levels of lidocaine could theoretically be reached within 7 minutes of application. A general increase in permeation throughout the period of experimentation can be noticed rather than post 2 hours as seen with LFS pre-treatment only, this could be due to LFS treatment occurring post MNs treatment allowing for further enhancement of the cavities formed via the MNs patch.

Figure 3.19 Different ultrasound output power with 10 min treatment time combined with 1.1 mm and 1.4 mm MNs patch (results represent mean ± SD values based on data from 6 skin samples).
Although following the same procedure bar the change to rotatory evaporation for the removal of residual paraffin oil and n-hexane a marked decrease in permeation was observed with F5. This could be due to the higher heating temperatures required to allow for sufficient organic solvent evaporation.

However a similar trend to that of F4 was observed (Figure 3.21), with a 10 minute application of 18W LFS yielding a significant difference after 1 hour (P<0.04) with an average 2 fold increase in permeation post 1 hour. A 5 minute application of the MNs array led to an initial 2.8 fold increase after which an average of a 3.4 fold increase was observed. Combining the two pre-treatments resulted in an initial 3.8 fold increase in permeation followed by an average 4.1 fold increase in comparison to passive diffusion only. Therapeutic levels of lidocaine were reduced from just over 2 hours to less than 1 hour on average.
Figure 3.21 NaCMC/GEL 1:2.66 (F5 PD), NaCMC/GEL 1:2.66 (F5 LFS, 18W 10min.), NaCMC/GEL 1:2.66 (F5 MN, 5 min.) and NaCMC/GEL 1:2.66 (F5 MN 5 min, LFS, 18W 10min).

### 3.4 Summary

The idea of combining sonophoresis with MNs patch provides a feasible way for the delivery of large molecules. The permeability of BSA, which has a relatively large molecular weight, is proved to be significantly increased as shown in this chapter. For the purpose of this chapter, the skin samples are pre-treated with both ultrasound and MNs patch for 10 min each. The BSA permeability reaches 1 µm/s which is a reasonable amount for delivering small dosage of the molecule. It also indicates the possibility of transporting large molecules through human skin in future. Molecule like insulin which is much smaller than BSA should have larger permeability if similar approach is used. The combination of MNs patch and ultrasound may become a painless alternative to the hypodermal injections for delivering large molecules. This study aimed to use low frequency sonophoresis and MNs as a pre-treatment to skin in order to enhance permeation of lidocaine encapsulated in a formulation. The greater stability was found with lower gelatine ratios (1:1.6) however all formulations were sufficiently stable (< -30mV). Viscosity results showed a pseudoplastic nature however no distinct pattern was observed between formulation and viscosity results. Diffusion
experiments revealed a significant increase in permeation when low frequency sonophoresis was used in combination with a MNs array as a pre-treatment. However rotatory evaporation during the synthesis stage caused significant reductions permeation levels. F4 and F5 were shown to decrease time required to reach therapeutic levels of lidocaine 5.7 & 2 fold respectively. Permeation showed a significant increase with higher sonophoresis power however increasing exposure duration demonstrated a minor increase. Whereas MNs application duration resulted in a significant increase in permeation. When combining MNs and low frequency sonophoresis pre-treatments the time to reach therapeutic lidocaine levels was significantly reduced, in the case of F4 therapeutic levels of lidocaine were reached within 7 minutes of application. Therefore this technique could be of medical use as a painless, easy to administer technique for drug delivery overcoming the time constraints usually associated with transdermal drug delivery.
Chapter 4
Roles of microneedles insertion force on drug permeability in skin

Overview

In this chapter, the permeation of large drug molecules (insulin, BSA) affected by different insertion forces on MNs will be discussed. Many experiments conducted in the literature have investigated the effect of MNs on insulin permeation across skin. There are also a number of papers focused on the insertion force required for MNs successfully piercing into skin. However, there is little known on quantifying the relationship between the effect of MN insertion force and the amount of insulin permeated for given MNs. In this chapter, we have addressed this subject by using 1100 µm and 1400 µm long MNs to conduct in vitro permeability experiments on porcine skin with insulin and BSA as the target drug molecules. Histological images of MN treated skin are obtained from a microtome and the viscoelastic properties of the skin sample are measured using a rheometer. An in-house insertion force device is utilised that can reproducibly apply a certain force on MNs for a set period of time maintained by compressed air. It is deduced that when porcine skin was pre-treated with an applied force of 60.5 N and 69.1 N. The resultant amount of insulin permeated was approximately 3 µg and 25 µg over a 4 hour period for the MNs used while the amount of BSA permeated was 4 µg and 12 µg under same conditions. The amount of MN force applied to porcine skin was shown to be related to the amount of insulin and BSA permeated. An increase in insertion force increase the amount of insulin permeated. It was also demonstrated that using insufficient force may have reduced or prevented the amount of insulin passing through the skin, regardless of the geometry of the MNs. Furthermore we compare the significance of force and ultrasound in increasing the drug permeability/concentration.

4.1 Background

MNs treatment is a well-developed technology in transdermal drug delivery which has been specified in previous chapters (Tuan-Mahmood, 2013). The MNs are aimed at minimizing mass transfer distance between the drugs and the blood micro-circulation by physically creating micron-scale channels in skin which let the drug molecules to transport through the skin over a shorter distance. The performance of the MN patch on enhancing drug delivery is based on three main factors. First, the inherent design of the MN patch which include the geometry (length, radius, shape) and the number of needles (MNs density) are important as they directly affect the drug permeability and the insertion behaviour (Al-Qallaf et al., 2009; Olatunji et al., 2012); Secondly, for a certain type of MN patch, the force applied on the patch will determine its
insertion behaviour and, hence, its performance. The insertion force is used to overcome a number of force components as discussed in detail by Olatunji et al (2013). An appropriate force can help the MNs to overcome the resistance of the skin where the resistance is typically determined by the viscoelastic property of the skin (Olatunji et al., 2013). It is known that the top layer, i.e., the stratum corneum (SC), is the dominating skin layer which governs the overall viscoelasticity of the skin (Yuan and Verma, 2006) and provides the maximum resistance to MN for the insertion process; Thirdly, the molecular weight of the drug molecule is imperative as it determines if it can pass through the SC passively. Large molecules may need sufficient penetration depth to achieve a reasonable permeation rate.

Insulin has a molecular weight of 5.8 kDa and is widely used in the treatment of diabetes. It cannot pass through skin passively due to its large molecular size but research shows that it can be delivered through skin with the treatment of different types of MNs. These MNs include dissolving, solid and hollow MNs are applied for both in vivo and in vitro studies (Norman et al., 2013; Martanto et al., 2004; Ling and Chen, 2013). An in vivo study on rats has successfully shown a great amount of permeation of insulin (Martanto et al., 2004). But, in an in vitro study, while the porcine skin has been chosen as typical samples for experiment, the permeated amount of insulin is much less than that in rat skin sample due to the higher thickness and stronger mechanical properties of the porcine skin (Garlanda et al., 2012). Because porcine skin is more structurally similar to human skin, the permeability enhancement for insulin in porcine skin poses great relevance in research. Unquestionably, different designs and patterns of the MNs patch can directly affect the drug permeation results (Al-Qallaf and Das, 2009). But another crucial factor in the enhancement of permeation is the force applied on the patch as mentioned earlier. A sufficient force is needed to ensure that the MNs are properly penetrated into the skin instead of only buckling the skin sample. Full penetration of MNs will need even higher force to fulfil which obviously will lead to higher permeation of drug molecules. It is expected that normal thumb pressure will be applied while inserting MNs into skin, but different humans are expected to exert different amounts of force while inserting MNs. Numerous researches have been conducted to study MN insertion force and its relationship to various parameters; such as tip radius (Davis et al. 2013), pain upon penetration (Hirsch et al., 2012) and penetration depths (Roxhed et al., 2007). However, there have been little or no studies conducted on the direct relationship between MN insertion forces and the permeation of large proteins, such as insulin. We address this issue and attempt to eliminate this knowledge gap by exploring this relationship; by relating the total insertion force acting on two different MN patches and the in vitro-permeation of insulin in porcine skin. As the insertion behaviour depends on the viscoelasticity of the skin, we determine the properties of these skin samples used in this work. Drug permeation is then related to the amount of force applied for a given MN patch. It seems, in order to reach the expected insulin delivery rate, a relatively high MN
insertion force is required for the skin samples used. We discuss these in more detail in the latter part of the chapter.

To achieve the purpose of this study, two different MN systems with varying lengths are used, which were done so as to confirm that the trend of results obtained from one particular MN length is observed for another length of MN. It is not the objective of this study to compare directly the performance of one MN array with the performance of another as the geometry of the MNs is different. The chosen MNs maintain continuity of some of our previous studies (Zhang et al. 2013; Nayak et al. 2013; Han and Das, 2013). We compare the significance of force and ultrasound in increasing the drug permeability/concentration.

4.2 MATERIALS AND METHODS

4.2.1 Materials

Actrapid® insulin (manufactured by Novo Nordisk, Kalundborg, Denmark) was purchased from a local pharmacy shop. Bovine serum albumin (BSA) and methylene blue were obtained from Sigma-Albrich (Gillingham, Dorset, UK). A reverse phase high performance liquid chromatography (RP-HPLC) instrument (Agilent Series 1100, Cheadle Cheshire, UK) was used to determine insulin concentration. Two reagents, acetonitrile and trifluoroacetic acid (TFA), were used as the mobile phases for the HPLC analysis. They were obtained from Fisher Scientific UK Ltd (Loughborough, UK). A Kinetex (length: 100 mm, internal diameter: 4.6 mm) column equipped with a Security Guard column (Phenomenex, Inc., Macclesfield, UK) was used to quantify the samples containing insulin. A manual Franz diffusion cell (FDC) (Logan Instruments Corporation, New Jersey, US) was used to conduct the permeation studies with and without MNs. Deionised water purified using a Millipore Elix System (Billerica, Massachusetts) was used for all FDC experiments. The skin samples used for the permeation studies were taken from porcine ears that were purchased from a local abattoir. The detailed preparation of the skin samples was the same as mentioned by Han and Das (Han and Das, 2013)). Two commercial MN patches, namely, AdminPatch® Array 1200 MN (1100 µm length) and AdminPatch® Array 1500 MN array (1400 µm length) (AdminMed, Sunnyvale, California, USA) were used to pre-treat the porcine skin. These patches have been proved to help passage of large molecules through the skin (Han and Das, 2013). The MN patches contain MNs of different dimensions and therefore, they are well suited for use in this work as they allow us to relate the MNs dimensions, force of insertion and the insulin permeability. There are 43 individual needles on the 1100 µm long MNs patch and 31 individual needles on the 1400 µm long MNs patch.
An experimental rig (device) was built in-house to apply a variation of MN forces onto porcine skin sample. All the components of this device were purchased from RS Components Ltd (Corby, UK). The device is discussed in more detail in the next sub-section.

Methylene blue was used for staining experiment and was purchased from Sigma-Aldrich (Gillingham, Dorset, UK). In combination with optical microscopes, a cryotome instrument (Bright Instruments Co Ltd, Huntingdon, UK) was used to ascertain depth profiles (histology) of the pre-treated and non-pre-treated skin samples. The viscoelasticity of the skin samples were measured by using a rheometer from TA instrument (New Castle, Delaware, US). Same ultrasound system (Branson Digital Sonifier 450 Danbury, CT, USA) as mentioned in previous chapter is used to apply sonophoresis on the skin samples.

4.2.2 Microneedles insertion force device

This study has used an in-house system which is designed based on double acting pneumatic pump (Figure 4.1) has been manufactured specifically to provide a reproducible force on MNs for insertion onto skin samples.

![Figure 4.1 The sketch of the double acting pneumatic pump: the gauge pressure is maintained by air pressure which can be controlled by the regulator. The rod is connected to the holder that attached to the MNs patch.](image)

The following are used to construct the device: a double acting 16x50 mm pneumatic air cylinder, an air pressure regulator and a directional control valve (5/2 spool valve). The developed system operates as follows. Compressed air pressure is used to activate the
pneumatic cylinder in the system, which acts like a piston. The imposed air pressure can be recorded which mimics the load applied on to the MN. The amount of compressed air running through the system is controlled by an air pressure regulator and it is operated by a spool valve lever. The system is fitted with a directional control valve that has 3 settings. When the lever is in the central position (neutral position), the internal shaft of the pneumatic cylinder is free moving as the spool valve is open and under atmospheric pressure. In this position the MNs can be attached to the MN holder and then placed on top of the porcine skin with no excessive force. When the lever is moved to the “on” position, the regulated compressed air is channelled to the top of the pneumatic cylinder. This forces the air piston to move down as the air flows through and maintains a constant force onto the porcine skin for a required time. When the lever is moved to the “off” position, the regulated compressed air is channelled to the bottom of the pneumatic cylinder, which forces the air piston to move up to the top, as the air flows through.

The imposed force and the MNs are perpendicular to the skin sample at the point where the MN contacts the sample. This enables us to define that the applied force is used to penetrate the MNs in the skin samples. Subsequently, the force (i.e. air pressure over active area) exerted by the insertion device on the MN patch is calculated using Equation 4.1.

\[ F = \frac{pm(d_1^2 - d_2^2)}{4} \]  

Where \( F \) is the force exerted (N), \( p \) is the air pressure (N/m\(^2\)), \( d_1 \) is the bore piston diameter (m) and \( d_2 \) is the piston rod diameter (m). The force generated by the pump should be considered as pure body load which is vertically orientated to the MNs base. In the scenario of penetration under high insertion forces, the MNs could be bent due to the deformation of the skin.

4.2.3 Permeability measurements using Franz diffusion cell

An aforementioned Franz diffusion cell apparatus (Figure 3.6) was used to measure the insulin permeability in skin. 70 µg of insulin containing solution was placed into the donor chamber and samples extracted from the receiving compartment at time points of 1, 2, 3 and 4 hours. The procedures for conducting the permeation experiments are the same as detailed by Han and Das (2013), and therefore they are not discussed in this chapter.

4.2.4 Microneedles insertion into porcine skin

The porcine skin in this study was not stretched when a force was applied. This was due to the various porcine thicknesses used, which would add variability in calculating the percentage
increase of the original size. This would be inadequately controlled, therefore may lead to variation in the tension of each sample. Therefore, it would be hard to keep the same tension under in-vivo conditions.

The force device mentioned in section 4.2.2 was used to apply a range of pressures onto the surface of the porcine skin (0.10, 0.20, 0.30, 0.35 and 0.40 MPa) which are converted to the total forces of 17.3 N, 34.6 N, 51.8 N, 60.5 N and 69.1 N, respectively. The forces were calculated based on a pneumatic cylinder used in the experimental set up. The equation used for the calculation is shown in Equation 4.1. The forces were chosen based on experimental results, in which a force increase was applied to allow a permeation of drug. As, the 1400 µm and 1100 µm AdminPatch® MN patches consist of 31 and 43 MNs on each patch, the force acting upon one individual MN is approximately 2.2 N and 1.6 N, respectively assuming that the applied force is aligned in the same direction as the MNs and perpendicular to the skin. Pre-excised porcine skin cut into 1.5 cm² pieces stored in a petri dish was placed below the MN. The MNs attached to the piston were then aligned on top of the porcine skin. The spool valve was set to the on position, where a constant force of MN was inserted into porcine skin for 5 minutes. The skin sample was then removed and placed onto the receptor chamber of a diffusion cell.

4.2.5 Method of analysing insulin concentration using HPLC

The concentration of the sample from the Franz diffusion cell experiment was analysed by RP-HPLC. The diode array detector (DAD) was set at 214 nm (Brix et al., 2002). A gradient method was adopted with eluent A: 0.1 % TFA in water and B: 0.1 % TFA in acetonitrile, with a mobile phase ratio of A: B, 95:05 to 05:95. The sample size of each injection was 10 µL. The temperature was set to 30°C. The flow rate was set to 1 ml/min. A complete RP-HPLC run took approximately 10 minutes with a down time of 2 minutes between each run. Calibration data of HPLC can be found in Appendix.

4.2.6 Sectioning of skin samples using cryotome

A depth profile (histology) of the pre-treated (MN) and non-pre-treated porcine skin was prepared using cryotome. The pre-treated skins were subjected to 1100 µm and 1400 µm long MNs with the insertion forces applied for 5 minutes. In this chapter 69.1 N insertion force was chosen to obtain a clearer cross sectional image of the skin sample and the holes created by the MN. Methylene blue dye was diluted with water to prepare a 50/50 (v/v) solution. The diluted dye solution was carefully poured onto the top surface of the skin sample and washed off after 3 min. Subsequently, the stained sample was cut into a 2x2 cm² pieces. The skin sample was then placed into the cryotome machine for 24 hours. The frozen sample was perpendicularly cut
through one row of MNs holes using a surgical scalpel, which is shown in Figure 4.2A, Figure 4.2B and Figure 4.2C depict the AdminPatch® Array 1500 MNs and AdminPatch® Array 1200 MNs. During this procedure, the cutting side was attached to the mould's wall to make sure that the section showed a real depth profile of MN holes that could be observed.

Figure 4.2 A: The MN pre-treated skin sample is cut through in a row using a scalpel, B: shows an image of AdminPatch® Array 1500, C: shows an image of AdminPatch® Array 1200.

The mould consisted of a rectangular plastic base and a glass baffle plate. The glass baffle plate was removed after the specimen was frozen. After the removal of the baffle plate, the frozen specimen could easily be removed from the base mould while keeping its structural integrity. The mould was filled with embedding medium (Bright Cryo-m-Bed, Huntingdon, UK) to 1 mm depth and kept in the freezer until the gel was completely solidified. The skin sample was then mounted onto the frozen layer with its cutting side sticking to the front inner wall of the mould. More embedding medium was filled until the whole skin sample was submerged. The whole mould was placed in the liquid nitrogen tank for flash freezing. The setup of the moulding section is shown in Figure 4.3.

The temperature in the microtome was set to -20°C. The angle of the blade was set to a normal cutting position of 15 degrees. The anti-roll guide plate was set parallel to the cutting bevel and has a 50 µm gap in between. The top surface of the guide plate was just above the blade's edge. The thickness of the slices was set to 15 µm. The specimen was glued onto the object hold using embedding medium and left in the chamber of the microtome until it was tightly bound to the holder.
4.2.7 Viscoelasticity of skin samples

In order to characterise the viscoelasticity of the skin samples, the storage and loss modulus of the skin were determined under oscillatory stress and a fixed strain. The storage and loss moduli are recorded at a volume strain of 1% with an increasing angular frequency from 6 to 474 rad/s (Holt et al., 2008). The storage ($G'$) and loss moduli ($G''$) shows the elastic and viscous properties of the material, respectively. They can be defined as:

\[
G' = \frac{\tau_0}{\gamma_0} \cos \delta \tag{4.2}
\]

\[
G'' = \frac{\tau_0}{\gamma_0} \sin \delta \tag{4.3}
\]

Where $\tau_0$ is the oscillatory stress, $\gamma_0$ is the strain (1%) and $\delta$ is the phase shift (10-20 degrees). The equations show that at a certain strain both the storage and loss moduli have proportional relationships with the stress. The amplitudes of these two dynamic moduli that refer to the energy storage and loss during the measurement can be used to describe the viscoelastic properties of the skin under different shear stress and shear rate. It indicates that if the storage modulus is high, there will be more resistant force from the material against the external forces.
The rheometer is set to an oscillating mode and the geometry of the plate is plain with teeth to make sure that there is no movement between the plate and skin sample during the test. The plate will move down toward the skin surface and stop when all the teeth on the plate are embedded in the skin. The plate is then start to oscillate with increasing angular frequency to generate different shear stress on the skin. With a fixed shear strain, the storage and loss moduli will be recorded as well as the phase shifts which are fluctuated within a range between 10-20 degrees (0 = pure elastic, 90 = pure viscous). The viscosity of the skin sample is also calculated from the loss modulus and it can be related to the shear rate using the following equations:

\[ \tau = \eta \dot{\gamma} \]  
\[ \eta = \frac{G''}{\omega} \]

Where \( \tau \) is the shear stress, \( \eta \) is the viscosity, \( \omega \) is the angular frequency and \( \dot{\gamma} \) is the shear rate. When the shear rate is increasing, a higher shear stress is always required as the viscosity should remain constant.

**4.3 Results and discussions**

**4.3.1 Characterisation of porcine skin**

Staining experiments were conducted on porcine skin to visualise that the needle insertion create holes in the skin samples. Figure 4.4A, B and C depicts an image of the porcine skin when it has been punctured by the 1500 AdminPatch® MN (Figure 4.4D). Figure 4.4E shows a schematic diagram of the spacing between 4 equidistant MNs. The results show that the needles have successfully pierced into the skin. The dimensions of the holes are shown to be: A-57 µm, B-50 µm and C-64 µm.
Figure 4.4 A, B and C shows 3 insertion holes created from 3 individual MNs using AdminPatch® 1500 MN array using 69.1 N insertion force. The diameters of the holes are: A: 57 µm, B: 50 µm and C: 64 µm, calculated using ImageJ. D shows a photographic image of AdminPatch® Array 1500. E shows a schematic diagram of the AdminPatch® Array 1500. It shows spacing between 4 equidistant MNs, spacing A: 1.97 mm, spacing B: 3.00 mm and spacing C: 1.55 mm, calculated using ImageJ.

As mentioned earlier, cryotome was used to visualise the cross section of porcine skin with and without MN pre-treatment. Methylene blue dye was applied to the surface to see what effect MNs have upon the SC. Figure 4.5A shows the cross section of porcine skin with no pre-treatment of MNs which, as expected, shows that the SC is not disrupted. Figure 4.5B shows a view where the SC has been disrupted when a force of 69.1 N was applied using the 1400 µm long MNs. The hole created by the MNs is evidently showing the SC layer is disrupted which suggests a noticeable increment of drug permeation. Figure 4.5C shows the same force being applied using the 1100 µm long MN patch, the depth is affected as it is shown that the holes created by the MNs are much more shallow. The 1100 µm MNs are smaller in sizes and a much higher MN density as compared to the 1400 µm patch. So it increases the insertion difficulty as well as it makes it less able to disrupt the skin sample. Figure 4.5D shows the results using the 1100 µm microneedles under 60.5N. It seems to indicate that the skin sample is more like being stretched and buckled rather than being penetrated by the MNs patch. The SC layer still partly maintain its integrity under 60.5N force, thereby lessening the diffusion rate.
Figure 4.5 A: Microscopic images taken of cryotome porcine skin (without MN), B: porcine skin pre-treated with 1400 µm long MN with a force of 69.1 N, C: porcine skin pre-treated with 1100 µm long MN with a force of 69.1 N and D: porcine skin pre-treated with 1100 µm long MN with a force of 60.5 N.

The shape of the hole is also more integrated because of the size of the needle and shows that during insertion the MN may have had bent.

4.3.2 Viscoelasticity of skin samples

The importance of considering viscoelastic properties during MN insertion has been discussed earlier, e.g., by Olatunji et al. (2012; 2013). The variation in the viscoelasticity of the skin is likely to affect the insertion behaviour of the MNs and, hence, it is best to consider the specific skin samples and their properties. As such, we discuss the viscoelasticity of the skin samples used in this work. The results in Figure 4.6 show that the skin samples are predominantly elastic as $G' \gg G''$. 

Figure 4.6 The average storage (G') and loss (G'') moduli of the skin samples with an increasing angular frequency (n=2).

The figure shows that the storage modulus is over 15000 Pa while the loss modulus is only around 5000 Pa. It also means that a high resistance to external forces is likely to be offered by the skin sample. This has been somewhat confirmed by our difficulty in inserting the chosen MN patch, particularly with the high needle density version (AdminPatch® Array 1200).

The viscosity is calculated using the loss modulus at every angular frequency. As the strain is set to 1%, the shear rate will be rising when the angular frequency is increasing. The resultant relationship between the viscosity and the shear rate is shown in Figure 4.7.
According to equation 4.4, the viscosity is related to the shear stress and shear rate during oscillatory measurement. However, the shear stress will be too low if the shear rate is under 0.2. Therefore, the rotation speed must reach to a certain value so the viscosity measurement can be stabilized.

4.3.3 Permeability of insulin

A variation of forces applied on MNs was conducted on porcine skin in vitro to ascertain the amount of insulin permeated when the MNs were used for pre-treatment. Porcine skin that was not treated with MNs was used as a control. Insulin Actrapid is a 5808 kDa molecule, which is considerably larger than 500 Da required for passive diffusion through the SC. Therefore no insulin is expected to permeate the skin when conducting passive diffusion experiments.

The amount of insulin permeated for passive diffusion and MN insertion forces of 17.3 N and 34.6 N was almost undetectable. Therefore no results have been reported in this paper. This may be due to the skin only being buckled or slightly pierced, therefore sufficient pathways cannot be created to let the insulin molecules pass through. Figure 4.8 illustrates the amount of insulin permeated when MN penetration forces of 51.8 N, 60.5 N and 69.1 N were used to pre-treat the porcine skin samples. The total amount of the insulin used for each diffusion experiment was 70 µg and the ratio of the insulin in the receiving compartment and donor compartment was shown to increase from 0.2 % to 37.1 %. The results show that after 4 hours the amount of insulin permeated was approximately 3 µg and 25 µg, respectively for 60.5 N and 69.1 N insertion forces, but were negligible when an insertion force of 51.6 N was applied.
There was almost an 8 fold increase in the amount permeated using the larger insertion force. More importantly, it confirms that an increase in the insertion force results in an increase in the amount of insulin permeated. The lack of insulin permeated when insertion forces less than 51.8 N were used, could be the result of the "bed of nails" effect.

Figure 4.8 Cumulative amount of insulin (μg) permeated with a MN insertion force 51.8 N, 60.5 N and 69.1 N for over 4 hours (n=3). The amounts of insulin permeated for MN insertion forces of 17.3 N and 34.6 N and passive diffusion were undetectable.

Although the SC layer is likely to have been disrupted, the high density MNs may not have been able to pierce through the whole epidermis due to the elastic properties of the skin, greatly affecting the permeation of insulin. This resulted in a lower concentration of insulin, compared to the less dense MN.

Figure 4.9 illustrates the amount of insulin permeated when 1400 µm and 1100 µm long MNs of penetration force 69.1 N was used to pre-treat porcine skin. After 4h, the amount of the permeated insulin with the 1400 µm patch pre-treatment was approximately 3.3 times higher than the 1100 µm patch. The ratio of total received insulin increases from 11% to 37.1%. It indicates that the geometry of the MN is also a crucial factor that can affect the insulin permeation. The length of the MNs is directly related to the diffusion pathway and is the main factor that determines the permeation of insulin. Our results also demonstrate that the insertion behaviour of the MNs, and therefore the hole sizes are affected by the viscoelastic property of the skin and the needle density on the patch. Although the insertion force is high, a higher density of the MNs can result in a shorter insertion depth than expected which leads to a lower permeation.
4.3.4 Permeability of BSA

The MNs enhanced permeation for the diffusion of BSA is also conducted by applying the same amount of forces (69.1N, 60.5N and 51.8N) for 5 min. However, the increment on the permeability of BSA solution is not significant as compared to the insulin permeability. The BSA solution in the donor compartment has concentration of 1000 ppm which equals to 2000 µg BSA of total amount. The results of cumulative amount of BSA in the receiving compartment for 4 hr duration under different insertion forces are showing in Figure 4.10.
Figure 4.10 Cumulative amount of BSA (µg) permeated with a MN insertion force 51.8 N, 60.5 N and 69.1 N for over 4 hours (n=3).

The cumulative amount of BSA permeated through the 1400 µm MNs treated skin with 69.1 N insertion force after 4 hr is only 11.7 µg which indicates ratio of the total amount of BSA in the receiving compartment to donor compartment is 1:171 (0.585%). It is a relatively low ratio comparing to insulin permeation under same conditions which shows a percentage of 37.1%. Although the permeability is increased by introducing higher insertion force, but the total amount of the BSA in the receiving compartment is still limited. The outcome of the enhancement from different insertion forces also suggests that the permeation is only significantly increased when applying 69.1N force on the 1400 µm MNs, but indistinct when lower insertion forces (51.8N and 60.5N) are employed despite of the size of drug molecules. However, 69.1N is a relatively high amount of force which may cause painful feelings to the patients. The feasibility of this amount of insertion force will request to be verified in clinical trials.
Figure 4.11 Cumulative amount of BSA (µg) permeated with a MN insertion force of 69.1 N for over 4 hours for 1500 and 1200 AdminPatch® MNs (n=3).

Figure 4.11 shows the results of BSA diffusion through 1100 µm and 1400 µm MNs pretreated skin under 69.1N insertion force for 4 hr. The total amount of BSA permeated through the 1400 µm MNs pretreated skin is 4.3 times higher than the amount when pretreated with 1100 µm MNs under same insertion force. This indicates that the length of the MNs is still affecting the diffusion results of large molecules like BSA. Both the size of the MNs and the insertion force are important factors for the enhancement of diffusion of large molecules. These results will prompt a new subject which is using those two elements as a synergetic system to optimize the diffusion rate of large drug molecules.

4.3.5 Comparing the effect of insertion forces with sonophoresis

Based on the experimental results of MNs enhanced BSA permeation using different insertion forces, the effectiveness of this method can be quantified. It can be used to compare with our previous study where we used ultrasound to further increase the skin permeability after MNs was applied with thumb pressure (17.3N). The BSA diffusion results of using 1100 µm, 1400 µm MNs under thumb pressure, sonophoresis with 3W, 6W and 9W output power are compared in Figure 4.12.
Figure 4.12 The BSA diffusion results only using different sonophoresis output power (3W, 6W and 9W) are compared with 1100 µm and 1400 µm MNs pretreatment under thumb pressure.

From the Figure 4.12, the results suggest the ultrasound effect on BSA permeation is insignificant at low output power with a maximum cumulative amount of 0.19 µg. Likewise, the length of MNs is not a strong factor in the enhancement of BSA permeation when the insertion force is low. However, the previous results on high insertion force is presenting a much stronger enhancement on the BSA permeability which is approximately 50 times higher than the enhancement using thumb pressure when the insertion force increases 4 times. We have explored the mechanism of MNs enhanced permeation combined with ultrasound in Chapter 3. Here, we want to compare some of the results from the ultrasound and MNs combination with the outcomes of high insertion forces. The first set is comparing the data of using the different powers of ultrasound (9W, 12W, 15W and 18W) to apply on 1100 µm MNs pretreated skin for 10min under thumb pressure with MNs alone under different insertion forces (thumb pressure, 51.8N, 60.5N and 69.1N).
Figure 4.13 BSA permeability results affected by different ultrasound output powers combined with 1100 μm MNs pretreated skin under thumb pressure and 1100 μm MNs alone with different insertion forces.

The results in Figure 4.13 show that the BSA permeability of 1100 μm MNs pretreated skin under 69.1N insertion force is 2.5 times higher than using 15W ultrasound combines with 1100 μm MNs. Although the increment of insertion force can provide more efficiency on TDD of BSA, but 69.1N is a relatively high force which not only requires great effort to generate, but can also cause the MNs to trigger the pain receptors in the dermis. Therefore, ultrasound combined with 1100 μm MNs as a guaranteed painless administration can provide reasonable amount of drug permeation even though higher diffusion can be achieved by increasing the insertion force.

The second set of data is comparing the effect of MNs length using both methods. The input of ultrasound powers and insertion forces are consistent only the length of the MNs is altered to 1400 μm. The results using 1400 μm MNs are shown in Figure 4.14.
The results indicate with higher MNs length, the enhancement of high insertion force comparing to MNs enhanced sonophoresis is more significant. The 1400 μm MNs under 69.1N insertion force can provide 6.6 times increment of permeability than using the MNs enhanced sonophoresis method. The intention of using 69.1N insertion force on 1400 μm MNs is not realistic because it will be certainly causing pain to patients. Comparing to the effect of insertion force, the enhancement of sonophoresis applied on the 1400 μm MNs pretreated skin is less significant. As a painless method, the permeability of BSA is doubled using ultrasound combined with MNs which is also commendable.

The last set of data intends to analyse the relationship between the insertion force and the effectiveness of sonophoresis on MNs pretreated skin. The results of 1100 μm, 1400 μm MNs under 51.8N insertion force alone is compared to the combination of 1100 μm, 1400 μm MNs under thumb pressure and 15W ultrasound treatment in Figure 4.15. The increasing rate of the cumulative amount of BSA using ultrasound enhanced method is constant due to the data is acquired at the end of the experiment. Therefore, the cumulative amount of BSA at each time point is calculated based on the average of concentration increment.
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Figure 4.15 BSA permeability results affected by 15W ultrasound output powers combined with 1100 μm, 1400 μm MNs pretreated skin under thumb pressure is compared with 1100, 1400 μm MNs alone with 51.8N insertion forces.

The conditions employed in this set of data are more acceptable. The ultrasound power is mild so it won’t cause pain to patients, but the insertion force is still relatively high. Both methods can provide reasonable amount of BSA in the receiving compartment which indicates these two method are all promising for further development.

4.4 Summary

MNs have been used to increase the permeability of countless drug molecules. The amount of MN force applied to porcine skin was related to the amount of insulin and BSA permeated. From the permeability experiments it is shown that an increase in insertion force increased the amount of insulin permeated through porcine skin. However, the rheological results of skin measurement suggests that the elasticity of the skin is 3 times larger than the viscosity so that a relatively high insertion force is needed to overcome the resistance from the skin. An insufficient force may not help the insulin or BSA to pass through the skin regardless of the geometry of the MNs. But, on a detectable level, the length of the MN and the force applied on the MNs are important factors that can greatly affect the permeation. By comparing the effect of insertion force with the enhancement of permeability using ultrasound combined with MNs, the results...
suggest the insertion force has more significant influence. However, the high insertion force will lead to a predictable consequence which is painfulness during the administration. Therefore, the design of the MNs patch and the insertion force must be optimized and considered as a synergetic system to reduce the risk of painful feelings.

As a scope for future work, it must be stated that more study on the effects of insertion force of MNs on the drug permeability should be conducted. For example, as an industrial application, the MNs used in this work could be used as a potential for sustained drug release, and if so, further studies on the clinical applications of these types of MNs should be conducted. Moreover, the insertion force has been proved as a crucial factor in the diffusion process. Hence, there is a great opportunity to optimize this factor incorporate with the MNs design in order to maximize the enhancement of ultrasound combined with MNs. The promising outcome of this idea is to achieve satisfied permeability whist reduce the risk of painfulness to the minimum.
Chapter 5
A new paradigm for numerical simulation of microneedles based drug delivery aided by histology of microneedles pierced skin

Overview

MN is a relatively recent invention and an efficient technology for transdermal drug delivery (TDD). Conventionally, the mathematical models of MN drug delivery define the shape of the holes created by the MNs in the skin as the same as their actual geometry. Furthermore, the size of the MN holes in the skin is considered to be either the same or a certain fraction of the lengths of the MNs. However, the histological images of the MN treated skin indicate that the real insertion depth is much shorter than the length of the MNs and the shapes may vary significantly from one case to another. In addressing these points, we propose a new approach for modelling MN based drug delivery, which incorporate the histology of MN pierced skin using a number of concepts borrowed from image processing tools. It is expected that the developed approach will provide better accuracy of the drug diffusion profile. A new computer program is developed to automatically obtain the outline of the MNs treated holes and import these images into computer software for simulation of drug diffusion from MN systems. This method can provide a simple and fast way to test the quality of MNs design and modelling, as well as simulate experimental studies, e.g., permeation experiments on MN pierced skin using diffusion cell.

5.1 Background

Transdermal drug delivery (TDD) methods, which intend to deliver various drugs through the skin, need to conquer the outermost layer of the skin, namely, the *stratum corneum* (SC) (Teo et al., 2006). Mathematical modelling of drug transport through the skin can provide important insights into TDD and it is considered to be important for analysing TDD as indicated by a large number of studies (Li and Zhang, 2014; Kalluri and Banga, 2009). This chapter aims to report a new paradigm for numerical simulation of MNs based drug delivery aided by histology of the MN treated skin.

The importance of the physicochemical properties of solutes for transdermal permeation has been known since the early 1940s. But, it is only since the 1960s, i.e., when Higuchi first related these properties to passive diffusion in percutaneous absorption of drug molecules (Higuchi, 1960) using Fick’s first law of diffusion that modelling diffusion of drugs for TDD has been seriously attempted. Based on the diffusion law, many researchers have now simulated drug
transport from different technologies that can enhance the TDD (Al-Qallaf and Das, 2009). MN
technology is a promising method and it is increasingly being explored for controlled
enhancement of TDD of different molecules (Kalluri and Banga, 2011; Roxhed et al., 2007).

The first MN modelling paper aimed to aid design of hollow MNs for fluid extraction (Brazzle et al., 1999). Since then, various researchers have focussed on improving the accuracy of
simulation by incorporating important fundamental features of the behaviour of MNs for drug
delivery, thereby, achieving better prediction of the drug diffusion behaviour (Olatunji et al.,
2012; Windbergs et al., 2013). For example, the geometry and size of the MNs, which are
important factors, have been considered in a number of previous modelling studies because
they determine the drug diffusion rate and flux from the MNs (Aggarwala and Johnston, 2004).
Consequently, optimization of the MNs geometry, shape and size of the MNs has been
attempted by many researchers, so that the desired TDD rate can be identified for specific case
(Al-Qallaf and Das, 2008). These frameworks demonstrate that the patterns of MNs and
alignment of these MNs on the patch are important to provide a sufficient delivery rate of drugs
(Al-Qallaf and Das, 2009).

Overall, it seems that the simulations for MNs based drug delivery are helpful for design of MNs
and understanding how they function but these require results of high quality and accuracy.
Generally, the current researches on MNs modelling idealize the size and shapes of the MN
holes (i.e., the computational domain) which are created by the MNs and often it is assumed
that the size of the holes are the same as the MNs themselves or a certain fraction of the
lengths of the MNs. However, the histological images of these holes indicate that the shapes of
MN and their associated holes may be very different (Groves et al., 2012). Not only the lengths
of the MNs pierced holes vary in length from case to case but also their shapes may vary. From
the point of view of TDD, this implies that the drug delivery rate should vary from case to case.
This can become a crucial factor that causes inaccuracy in the simulation results. For these
reasons, this chapter aims to develop a new paradigm in numerical simulation for drug delivery
by MNs which can incorporate the shapes and sizes of the MN holes as determined from the
histological images of MNs treated skin. It is hypothesised that more realistic simulated drug
transport behaviour (e.g., transient drug concentration profile) can be obtained by averaging
simulation results from a number cases given by different histological images for the same
circumstance (e.g., the same skin, MN length and drug molecule), coupled with experimental
data (e.g., diffusion coefficient of the drug or drug permeability) for the corresponding case.

In addressing this issue, this chapter introduces a new approach that should improve the
simulation accuracy of MNs based drug delivery. We present a numerical model based on the
histological images of MNs treated skin instead of using the conventional method (Al-Qallaf et
al., 2007) which considers the geometry of MNs in conjunction with a correction factor to
determine the lengths of the holes. There are several advantages of using these images, as discussed below.

When we consider the histological images and couple them with experimentally determined parameters (e.g., drug diffusion coefficient) for modelling the drug transport, the deformation of the skin due to insertion of MN (Olatunji et al., 2012; Olatunji et al., 2013), which affects the drug transport behaviour, will be directly accounted for. Most MN drug delivery simulations tend to ignore the effects of skin deformation as it is governed by the geometry of the MNs, force exerted on the MN patch and viscoelastic properties of the skin, thereby, making skin deformation and its effect on drug transport a difficult quantify to determine. However, there are several researches which focused on studying the factors that can cause skin deformation, e.g., the MN insertion force has been decomposed into several components to increase the accuracy of the simulation (Olatunji et al., 2013). Similarly, the viscoelastic properties of the skin layers have been considered (Shuhu et al., 2012). The histological images provide a view of the holes created by MNs on skins. For the same cases, experimental data on drug permeation and effective diffusion coefficient can be obtained. The accuracy of the information can also be increased by acquiring a number of these images and experimental data. As stated earlier, it is hypothesised in this paper that these information can then be used to carry out more accurate numerical simulations for MN based drug delivery.

The histological image can provide an efficient way to evaluate the practicability of the MNs modelling and, the images may be acquired irrespective of the source or the method of acquiring them. For example, the cross-sectional view of the MNs treated skin can be acquired by using a cryotome and then viewed under a microscope (Kalluri et al., 2011). Similarly, technologies such as optical coherence tomography (OCT) (Larrañeta et al., 2014; Kim et al., 2009) and micro-CT (Cai et al., 2014) have been useful in obtaining images of MN pierced skin. These images can show whether the pathways (holes) created by the MNs are adequate in overcoming the SC and for a target drug molecules to pass through.

In order to analyse the drug transport behaviour based on the histological images, a MATLAB program has been developed in this work which can automatically acquire the coordinates of various points of the image of the skin sample. These coordinates (i.e., not the image) can then be imported into a simulator (e.g., commercially available software COMSOL (Littmarck, 1986)) to carry out the desired simulations for drug transport.
5.2 Histology of microneedles treated skin as computational domain for numerical simulation

5.2.1 Acquisition of images of microneedles pierced skin

In this work, the computational domain for the numerical simulation is based on the histological images which can expose the cross-sectional views of MNs treated skin. The images are collected using porcine ear skin purchased from local abattoir (Han and Das, 2013); however, the developed methodology is general and it would work with images collected via any other means. The skin samples are freshly harvested and flash frozen using liquid nitrogen after sectioning and kept in the freezer at -20ºC if they are not used immediately after collection. The sample are wrapped in aluminium foil and left in room temperature to defrost for 2 hr before an experiment. The skin are cautiously separated from the underneath cartilage using a surgical scalpel.

Figure 5.1 A cryotome setup which was used to acquire the histological images of the MN pierced skin. The skin sample is wrapped in glue, fixed on the sample holder between the blade and anti-roll guide plate, and sliced into think sections.

1100µm/1400µm long MN patches which are purchased from nanoBioSciences (Sunnyvale, CA, USA) are used to pierce the skin. The real insertion depth of these commercial MNs patches are much shorter than the original length which has been demonstrated in our previous paper (Nayak et al., 2014). A MNs patch is pushed with a relatively high thumb pressure to ensure that all the needles are pierced into the skin and then mounted to a pneumatic pump with a constant 1MPa pressure for 10min. The sectioning protocol for the prepared skin sample using
cryotome (Figure 5.1) is consistent with our previous study and is not discussed in detail in this chapter (Cheung et al., 2014). The sample of sliced skin is then analysed under a microscope and a number of images are taken using a camera which is attached to the microscope. The acquired images are processed for numerical simulation as discussed below.

5.2.2 Processing of skin histological images for numerical simulations

An image of the skin histology cannot be directly used as a computational domain for numerical simulations as the skin layers/surfaces needs to be identified in terms of co-ordinate points. Furthermore, due to the possibility of a number of other factors which affect the quality of the original image for simulation purpose, the developed algorithm is designed to eliminate these factors. These are discussed separately in the latter part of the chapter.

Once the images of the cross-sectional view of the MN pierced skin samples are captured, all the images are imported into MATLAB (Natick, MA, USA) for processing (MathWorks, 1984). In this work, an in-house MATLAB program was developed to process these images. The first step of this program is to acquire all the edges of the image. To achieve this, all RGB images (conventional format using red, green and blue added on each other to reproduce all colours) need to be converted into grayscale images. A Canny edge detection algorithm (Canny, 1986) is then applied on the grayscale images to acquire the edges of the individual images. A Canny algorithm is an advanced image filter based on a Gaussian filter (Canny, 1983). It involves four stages, namely, (i) noise reduction, (ii) edge detection, (iii) edge thinning, and (iv) thresholding with any hysteresis, which provide high quality edges of computational domain in a black and white map. Two examples of images of skin pre-treated with MN, which have been processed with the Canny algorithm using the developed MATLAB code, are shown in Figure 5.2. From the figure we can see that the edges of the original images are acquired with high quality details. However, in reality, we do not need all the details in different skin layers as well as the debris outside the skin which do not have any significance for the drug transport. Furthermore, the presence of debris may influence the numerically determined transport behaviour. Therefore, the images obtained are further processed by specific algorithms to acquire smoother profiles of the skin layers/surfaces.
Figure 5.2 Two examples of histological images of skin pierced with MNs (images on the right) which are treated with the Canny edge detection algorithm (images on the left) for further processing and numerical simulations.

A1 Different steps adopted for smoothing the images of skin histology for numerical simulation

The algorithm for preparing the geometry of the computational domain for numerical simulation is consisted of four stages: (i) the image dilation and fill, (ii) debris identification, (iii) debris elimination and (iv) importing domain as computational domain for numerical simulation. When all four stages are finished, the acquired geometry of the domain is expected to be ready as a computational geometry for numerical simulations.

A1.1 Dilation and filling

Firstly, the edges acquired from the edge detection process need to be dilated to connect any gaps on the skin surfaces. It is a necessary step to accomplish an intact domain for numerical simulation from discrete lines as shown in Figure 5.2 (Svoboda et al., 2007). From a number of trials we have concluded that a 5 pixels×5 pixels dilation matrix is sufficient to connect all the gaps in the images that we have collected. However, this matrix may be different in another case where the image of the skin histology is significantly more complicated and requires significant amount of dilation and filling. To achieve higher accuracy, the size of the matrix,
A1.2 Debris identification

As discussed above, after the computational domain (an image) has been confirmed, there may still be some undesired skin debris in it. The debris can be hard to predict in the images because they can be constituted by loose debris of the skin sample (e.g., generated while the skin is pierced) as well as by other impurities or bubbles in the embedding compounds (e.g., gels used for acquiring cryotome images). The MATLAB program has been developed to automatically identify all kinds of debris so as to smooth the images irrespective of their sources.

For the purpose of debris identification, the debris in the image is discriminated from the main body of the skin sample and the debris is identified individually by the program. After the debris is identified, an elimination program is launched to remove the debris. This stage of the developed program is designed to remove debris from the image without causing any damage to the computational domain of interest. Our method shows great advantages comparing to other filtration methods (such as using rank or Gaussian filtering methods to remove debris) which can cause great damage to the outline of the skin (Solomon and Breckon, 2010). Further details of the debris elimination program will be introduced in section 5.2.2.1.3.

A1.2.1 Specifying corners of the debris in terms of co-ordinates

The debris identification stage is based on the assumption that the debris has closed edges/boundaries. In other words, if a point on the edge of debris moves along its edge, it will eventually come back to the same point where it started. This is an important factor in the developed algorithm so as to discriminate the debris from the skin sample and confirm which of the target debris is to be eliminated. If we choose a corner of the debris as the starting point, this point will then go from corner to corner and finally reach the original corner. Therefore, we need to classify the moving patterns between the corners. To explain this process a logic diagram is shown in the Figure 5.3. In the figure, the capital letters L/R/U/D/T/B/P/N represent the initial alphabets of words left/right/up/down/top/bottom/positive/negative, respectively, which represent how a position moves in the algorithm to identify the debris. For example, the combination of LTP indicates the following: (1) the current point is on one of the left (L) top (T) corners of the image; (2) the points on its lower right have their pixel intensity values equal to positive (P) 1.0; and (3) positive represents the direction of the edge detecting process passing...
this point is clockwise (while negative represents counter-clockwise). In contrast, the LTN points satisfy the inverse of the above three conditions for LTP points. A schematic diagram to explain the differences between the LTP and LTN points has been shown in Figure 5.4.

**A1.2.2 Principles for the identification of debris**

In this section, we discuss the principles and the mechanism of the debris identification process in more detail. The developed MATLAB program starts from LTP which could be a positive left top corner of a domain of any shape. To define a corner in an image, we define that a minimum number of four points are required. In this specific case at point (x, y), a LTP can be confirmed if it satisfies the following four conditions: \((x + 1, y - 1) = 0\); \((x, y - 1) = 0\); \((x - 1, y - 1) = 0\) and \((x - 1, y) = 0\) (for a 800x600 image, the coordinate from left top to left bottom is \(x = 0\) to \(x = 600\), from left top to right top is \(y = 0\) to \(y = 800\)). Once a LTP point is chosen, there are two initial conditions that should be satisfied for this program. Firstly, LTP is chosen as the only criterion being both the starting point and terminal point, and secondly, it is defined that the first move from LTP is always to the right direction (clockwise). From Figure 5.3 we can see that there are only two destinations if a LTP point moves to the right following the edges, i.e., RTP and RBN. If a point reaches the RTP, then it can only move downward to either RBP or LBN. An example edge detection process has been shown in Figure 5.5.

Although there are 8 different corners involved according to Figure 5.3, they are all connected to the four terminals which are marked with different colours. They are LTP/RTN/LBN/RBP marked with red/purple/blue/green, respectively. However, only the red LTP is the real terminal. When the process reaches red LTP, a check must be made to compare the current point to the original one. If they are the same point, it indicates that the edge detection process has been done because the selected point has travelled the entire surface of the debris and back to its original position again. If not, the process will go back to LTP1 and continues until it reaches the original point. The circle enclosed number represents pathway 1 which is connecting LTP to either corner RTP or RBN. These pathways can keep the edge detection process to stay on the edge. Otherwise, the process may never move from one corner to another.

When the edge detection process reaches other three terminals, it will jump back to the previous junction that has the same colour with that terminal, for example, all purple RTN terminals will jump back to junction purple RTN6. An illustration of a simple loop starting from junction RTN6, which moves to terminal RTN following the edge of the shape and then jumps back to junction RTN6, has been shown in Figure 5.6. Where the blue and black arrows represent the process conducted in logic diagram and reality, respectively. When the process jumps back to the junction RTN6, the point will move to the left again until it reaches the
terminal LTP. All three coloured junctions connect to a LTP terminal to avoid falling into infinite loops.
Figure 5.3 A logic diagram of edge detection algorithm: L=left, R=right, U=up, D=down, T=top, B=bottom, P=positive, N=negative. Circle enclosed number represents pathways connected to specific corners.
A1.2.3 The definition of hidden corners

In some cases, there are corners which are not easy to distinguish from the shape because the definition of a corner in this program and it may not be possible to assign co-ordinates precisely to each point. This is because these corners are concealed in the shape or overlapped with other points. However, they can be found by the edge detection process. The reason for the existence of these hidden corners is that the trajectory of the edge detection process is not always straight. When the trajectory is terraced, the corner will appear as irregular. An illustration of hidden corners RBN and RTN in edge detection process has shown in Figure 5.7.
Figure 5.6 An example of a typical case in which the debris identification process reaches a terminal (see Figure 5.3) where it will automatically jump back to its corresponding junction (each square in the diagram represents one pixel in the image).

Figure 5.7 Illustration of two hidden corners existing in this example figure, which are RTN and RBN formed due to terraced edge (each square in the diagram represents one pixel in the image).

The corners LTP and LBP are easy to identify but the corners of RBN and RTN are likely to be anomalous. Nevertheless, the RBN and RTN can be recognized by the program, and therefore, the developed MATLAB code will ensure that the edge detection process will not be affected.
A1.2.4 Special cases in the process of identifying corners

A special circumstance needs to be clarified when the edge detection process runs back to LTP from the only two possible corners: LBP and RBN. Three representative cases have been shown in Figure 5.8:

![figure showing three special cases](image)

Figure 5.8 Three special cases in the edge detection process which need to be identified individually in the program by the user (each square in the diagram represents one pixel in the image).

The first and second cases in Figure 5.8 show that when the edge detection process moves up from LBP and RBN to LTP corner, the program will not be able to recognize the LTP corner according to the logic diagram. The program will ignore the LTP corner and move on to follow the dashed arrow. The third case shows an extreme circumstance in which the LTP corner overlaps the LBP corner and the program will go on following the dashed arrow. The reason for this situation is due to the fact that when we define the LTP corner, we have used four points. However, only three points have been used to profile the pathways. The problem for all other corners has been solved by applying the ‘hidden corner’ concept which has been mentioned previously. But the LTP corner is different because the program may have to be terminated here. To solve this special case, an additional step must be introduced to compare the current LTP corner to the original one. Once the left top corner is confirmed as the same one of the original corner, it indicates that one of the debris has been identified so that the corresponding elimination process will be triggered.

A1.3 Debris elimination

Once all the debris has been identified, the program will jump to the elimination stage automatically to remove the skin debris. As mentioned before, a conventional image filter cannot be applied here due to the inaccuracy (Baxes, 1994). Therefore, the elimination process is specially designed which shares the same logical diagram with the debris identification process. However, the elimination process will erase every point that the moving point Y has passed through. Therefore, the program will not stop until the last point
of the debris is eliminated because the starting point has already been erased at the beginning. The elimination program peels off the debris shape layer by layer and jumps back to the debris identification program once the process is finished. An illustration of the elimination process is shown in Figure 5.9.

![Figure 5.9 An illustration of the debris elimination process (each square in the diagram represents one pixel in the image).](image)

The elimination process begins from the starting point which is a LTP corner of the shape and will be at the middle point of the shape that marked in light grey colour. However, the elimination process may not be able to finish in one go because sometimes the shape will be broken into several small blocks. It happens when the connection point is erased so that the moving point Y cannot go back to the original block to finish the elimination process. To solve this problem, the identification process is always scanning line by line from top to bottom. Once the first starting point is identified, the program will jump to elimination process
to remove the debris. After the elimination, the identification program will scan the next line and find the second starting point to remove the remaining part of the debris. The illustration is shown in Figure 5.10.

To increase the speed of the program, a global parameter ‘si’ is set to describe the maximum size of debris. The ‘si’ parameter is pre-set to 30 in this work, which means that the maximum size of debris in an image is equal to or smaller than 30x30 pixels. After all debris are identified and eliminated, the program will move to the next stage and the treated image compared to the untreated image is shown in Figure 5.11.

5.3 Numerical simulations using processed image

5.3.1 Acquiring processed image in numerical simulator

After the debris identification and elimination process, the image shows clear outline of the skin sample without the interference of the debris. The program then captures the profile of the skin by picking up all the points on the boundary. The thickness of the profile is defined to be one pixel and the acquired profile will be saved in a matrix.

After the profile of the skin surface has been acquired, the computational domain is converted into computational geometry whose boundary is consisted by coordinate of points (instead of boundary lines as seen in an image). This imported geometry is then meshed for numerical simulation. An example of a numerical mesh is shown in Figure 5.12. The coordinates of all points in the mesh are saved in a .txt file by the program, so the simulation can be done using any software. There is only one task that needs to be done by the users themselves, which is to find the ratio between the pixel and scale of the actual image. This requires user to set up a scale when they obtain an image using camera, micro-scope, OCT or micro-CT or any other source. After the scale is known, the ratio can be easily calculated by an image processing software.
Figure 5.11 Two pairs of typical images before (left) and after (right) treating with debris identification and elimination stages. The two images on the right hand (top and bottom images) side are now ready to be used as computational domains for numerical simulation.

Figure 5.12 A typical image of a histological image which is meshed for numerical simulation.
5.3.2 Governing equations

As discussed earlier in our papers, an effective skin thickness can be defined for MN treated skin which represents the average path length for drug diffusion through the skin (Davidson et al., 2008; Al-Qallaf et al., 2009). Using the same concept, we calculate the effective thickness in this work using the images that we have collected. We define that there is negligible change on the diffusion coefficient in viable epidermis due to insertion of MNs and the diffusion profile of drug molecules passing through the skin at steady state is obtained using the Fick’s first law:

\[ N_i = -D_i \nabla C_i \]  

(5.1)

Where \( N_i \) is the flux, \( D_i \) is diffusion coefficient, \( C_i \) is the concentration. In the simulation of MNs treated skin and the thickness of the skin change, so the effective thickness of the skin is introduced in consistent with our previous studies (Davidson et al., 2008; Al-Qallaf et al., 2009). Therefore, we deduce the diffusion coefficient from the experiment data of passive diffusion and the effective thickness in this study can be simply considered as reduction of the real skin thickness. The real skin thickness is 1.6 mm which is the average over 20 randomly selected samples. An effective thickness of the MNs treated skin is then calculated using equations (5.2) and (5.3) as discussed earlier (Al-Qallaf et al., 2009).

\[ D_{PD} = \frac{J_{PD}h}{\Delta C} \]  

(5.2)

\[ h_{eff} = \frac{D_{PD}\Delta C}{J_{MN}} \]  

(5.3)

Where \( J_{PD} \) is the flux from passive diffusion, \( J_{MN} \) is the flux from MNs treated diffusion, \( D_{PD} \) is the diffusion coefficient of the skin and \( h_{eff} \) is the effective thickness of the MNs treated skin.

We use the steady state model to analyse the experimental data because it is impossible to know the drug concentration at any position of the skin at any time point. However, the numerical simulation is able to provide simulated data at any specific time point and depth (Chaskalovic, 2008).

Once the effective skin thickness has been identified, we apply Fick’s second law in order to build a transient drug diffusion model:
\[ \frac{\partial c_i}{\partial t} + \nabla \cdot (-D_i \nabla c_i) = R_i \]  

(5.4)

Where \( c_i \) is the drug concentration, \( D \) is the diffusion coefficient, \( t \) is the time point and \( R_i \) is a constant.

The 1D boundary conditions to solve the differential equations (5.1) and (5.4) are:

\[ C = C_1 \text{ at } x = 0 \text{ (for all } t \text{ for transient drug transport)}, \]
\[ C = C_2 \text{ at } x = h \text{ (for all } t \text{ for transient drug transport)}, \]
\[ C = 0 \text{ for } 0 < x < h \text{ (at } t = 0 \text{ for transient drug transport)} \]  

(5.5)

Where \( C_1 \) is the constant drug concentration we give on the skin surface, \( C_2 \) is the drug concentration at the bottom of our skin model, \( x \) is the distance between skin surface and a specific point in the skin, \( h \) is the thickness of the skin. For transient simulations, \( C_2 \) is set to zero at the beginning and its cumulative values over time will be calculated using equation (5.4) until steady state is reached. In order to solve 2D and 3D differential equations, additional boundary conditions can be applied to equation (5.1) and (5.4):

\[-n \cdot N_i = 0 \text{ at plane } y = 0, l \text{ and } z = 0, w \text{ (at all } t \text{ for transient drug transport)} \]

(5.6)

Where \( n \) is the normal vector to the plane, \( N_i \) is the flux, \( y \) is the length between the front surface of the model and a specific point in the skin in longitudinal direction and the total length is \( l \), \( z \) is the width between the left surface of the model and a specific point in the skin in horizontal direction and the total width is \( w \). The governing equations are implemented in commercial software COMSOL by importing the coordinates of the points on the skin surface and choosing the element sizes for different accuracy scales of FEM mesh. The parameters that are needed for the simulations can be either acquired from experimental data or using the theoretical values (e.g., correlations). If the transient model is applied which refers to equation (4), COMSOL can show the diffusion results at any time point before it reaches the steady state.
5.4 Results and discussion

5.4.1 Calculation of microneedles insertion depths based on histological images

Four histological images of MNs treated skin samples have been chosen for both 1100µm and 1400µm long MNs. The insertion depth of each image is then calculated using ImageJ software (Bethesda, MA, USA) (Rasband, 1997) and the mean insertion depth of each group has been calculated. It is found that the average insertion depth of 1100µm and 1400µm long MNs are 0.53±0.054 mm and 0.7435±0.099 mm, respectively.

The theoretical insertion depth refers to the length of the MNs which are 1.1 mm and 1.4 mm, respectively. The full skin thickness is a constant value of 1.6mm, which is mentioned in the previous section.

The experimental data are obtained from our previous study effects of MNs on the permeability of insulin in skin (Cheung et al., 2014). Due to any drug molecules that larger than 500 Da cannot pass through the SC layer passively (Brown et al., 2006), we assume the diffusion coefficient for the diffusion of insulin (5808 Da) in the SC layer is negligible. The histological images also indicate that our MNs only partially removed the SC layer but the distance between the drug solution and the blood circulation is reduced. Therefore, the complex situation where the SC layer is involved will not be discussed in this chapter. Instead, we consider our skin model as one layer and only use the diffusion coefficient of viable epidermis to compare the passive and MNs enhanced diffusion. Based on those data from our previous study and our assumption, the diffusion coefficient (D_{PD}) is calculated as 1.3e-12 m²/s and the effective thickness of both MNs are deduced accordingly by applying equations (2) and (3), respectively. The insertion depths and skin thickness results acquired for insulin are shown in Figure 5.13.

As shown in Figure 5.13, we compare the full skin thickness with the calculated effective thickness of the skin, theoretical insertion depths (i.e., length of individual MN) and average MN insertion depths based on histological images. The results show that the effective thickness (and hence, the mean path length for drug diffusion) is larger than the MN insertion depths based on the images but smaller than the MN lengths or real skin thickness.
Figure 5.13 Two sets of comparison: full skin thickness compared to the effective thickness after MNs insertion. The theoretical insertion relates to the insertion depth calculated from histological images. The effective thicknesses were calculated using data from insulin permeation study for 1100µm (top) and 1400µm (bottom) long MNs.

The second set of experimental data comes from our previous study on the transdermal delivery of lidocaine carboxymethylcellulose with gelatine co-polymer hydrogel (Nayak et al., 2014). However, we only performed the 1100µm MNs with different time (3min and 5min) durations to analyses the drug diffusion in this study. The calculations are based on the controlled release study and the diffusion results in the paper from where the diffusion coefficient is calculated as 1.86e-10 m²/s. The calculated effective thickness of different treatment time are compared to full skin thickness, the theoretical insertion depth and average MN insertion depths based on histological images in Figure 5.14.
Figure 5.14 The histological insertion depth compares to the calculated insertion depth from lidocaine permeation study of 1100µm MNs treatment for a time duration of 3min (top) and 5min (bottom), respectively.

5.4.2 Numerical simulation of drug permeability/diffusion

B1 Testing developed scheme for an ideal microneedles geometry

In order to test that the developed algorithm performs, we attempt to simulate an ideal geometry of MN (Figure 5.15A) and define that the holes created by them in the skin match their size and shape. Therefore, the MN holes are well defined in terms of shapes and size (Figure 5.15) and have no debris in the image, making them a good model to test that the developed scheme is working. As obvious, in this case, the modelling does not involve the use of a histological image and the simulation is done using the image of the MN geometry.
(0.5mm length) with some bends and complex features. We define that such a MN system has been applied according to a ‘poke and patch’ approach where the MNs have been applied on the skin, removed after creating the MN holes and a drug is then applied on the skin which penetrate into the holes. The drug molecule then diffuses into the skin through the viable epidermis. In effect we simulate the drug permeation study in a typical Franz diffusion cell as discussed earlier (Han and Das, 2013).

The data for permeation of insulin from our previous experiment using Franz diffusion cells (Cheung et al., 2014) have been chosen to carry out the numerical test. The drug concentration on the skin surface is set to a constant value of 1000ppm and the bottom surface of the skin is set as zero concentration which indicates a static system such as those seen in the receptor compartment in a Franz diffusion cell. The time duration insulin is defined to be 48 hr for insulin and 4 hr for lidocaine.

Figure 5.15, Figure 5.16 and Figure 5.17 show some of the results obtained for the case above. In Figure 5.15 (C, D) we show the mesh used for the numerical simulation and concentrations distribution in the MN treated skin at steady state. Figure 5.16 shows the numerically calculated insulin concentration in the receptor compartment of a Franz diffusion cell with and without MN. The results indicate that insulin (molecular weight: 5808 Da) reaches 10% of its maximum concentration in the blood stream after 48 hr. It has also shown that MNs cause great effects on large size molecules because the trend lines between MNs enhanced diffusion and passive diffusion are distinct.

The results in Figure 5.17 indicate that lidocaine can reach its maximum concentration in the blood stream in quite a short time duration due to its small molecular weight (234.34 Da). The results have also shown that MNs produce less efforts on smaller size molecules because the trend lines between MNs enhanced diffusion and passive diffusion are obscure. However, the enhancement of MNs are remarkable for large molecules like insulin.
Figure 5.15 The process after the image is input into the program. A) The original image; B) The outline of the skin has been captured; C) The skin thickness has been adjusted to 1.6mm and the mesh using FEM has done; D) the simulation of the diffusion profile of the target drug molecule (insulin, in this study).
In this section, we will illustrate modelling using the image acquired from cryotome sliced skin sample captured by a microscope camera. The original and processed images are shown in Figure 5.18. The simulation is then employed based on those 2D histological images. The MNs we applied are flat in structure with negligible thickness, thereby 2D images are sufficient for this study. The same initial and boundary conditions as discussed earlier are again applied for the purpose of this section. The numerical results for insulin concentration in the receptor compartment of a Franz diffusion cell are shown in Figure 5.19.
The insulin delivery has been shown higher amount of drug permeated and reduced lag time (time from initiation to the steady state). The simulated data from all the histological images are now averaged and compared with the experimental results of insulin from our previous study (Cheung et al., 2014). The initial concentration of the drugs and time durations of the simulation remain the same with the experiment condition. The results are shown in Figure 5.20. From the figure we can see that that the predicted concentration of insulin resembled well to the experimental results.

Figure 5.18 The image from cryotome sliced skin is processed by program and simulated in COMSOL. A) The original image; B) The outline of the skin has been captured; C) Simulated diffusion profile of the target drug molecule (insulin, in this study).
Figure 5.19 The diffusion profile of the passive diffusion and MNs enhanced diffusion based on the imported model. The concentrations at the blood stream of insulin have been calculated.

Figure 5.20 Numerical simulation data compared to the experimental results from our previous papers (Cheung et al., 2014). The concentration profiles of insulin are presented.
Figure 5.21 The diffusion profile of the passive diffusion and MNs enhanced diffusion of lidocaine based on the imported model.

B3 Numerical simulation using images obtained from the literature

The third modelling scenario is based on a complex OCT image which we have collected from a published paper (Enfield et al., 2010). The OCT image has much lower resolution level than the images taken by the in-house camera in this study. Despite this, it is observed that the developed algorithm can depict the outline of the skin surface without losing significant details. The numerical modelling results using these images have been shown in Figure 5.22. The image in this case shows that the MNs create lower insertion depth but more holes than previous images. Assuming that the MNs in this example are used to carry out an insulin permeation study, the same boundary conditions are applied again on this image. The insulin concentrations in the receptor compartment of a FDC are shown in Figure 5.23. We also use lidocaine as second target drug molecule to show the concentrations profile in the receptor compartment of a FDC which is presenting in Figure 5.24. The concentration profiles indicate that although the numbers of the holes increased as MNs are pierced into the skin, the diffusion rate is low compare to the passive diffusion. The profiles of concentration between passive diffusion and MNs enhanced diffusion for both drugs are most close to each other comparing to previous two MNs modellings. This also suggests that simply increasing the number of the MNs will not greatly increase the permeability in this case. The insertion depth and the geometry of the MNs may present higher impact to the diffusion rate.
Figure 5.22 The OCT image is processed by the program and simulated in COMSOL. A) The original OCT image; B) The outline of the skin has been captured; C) the simulation of the diffusion profile of the target drug molecule (Enfield et al., 2010).

**B4 Numerical simulation of insulin permeation in 3D model**

The last case is an extension from the first scenario in order to demonstrate simulation of 3D structured MNs. There are plenty of studies from previous papers using 3D MNs model to simulate diffusion in TDD (Loizidou et al., 2014). However, our 3D model is constructed from the pores created by MNs instead of building a model using the shape of MNs. Different slices of histological images from one pore are combined together to form a 3D model and the accuracy will keep increasing when more slices are involved. In this case, we choose the sample shape from Figure 5.15 as the standard to maintain the consistency and illustrate the process by including another 8 random designed slices to construct the 3D model. The
profile of each sample slice is acquired using previous method and then all slices will be imported to COMSOL as individual surfaces which are shown in Figure 5.25:

Figure 5.23 The diffusion profile of the passive diffusion and MNs enhanced diffusion based on the model of Figure 5.22. Insulin concentrations at receptor compartment of FDC have been calculated.

Figure 5.24 The diffusion profile of the passive diffusion and MNs enhanced diffusion of lidocaine based on the model of Figure 5.22.
Figure 5.25 The sample slices have been acquired individually and then imported into COMSOL. A) The profile of the sample slice from Figure 4.15 which is located in the middle of all slices; B) Another sample slice located next to the standard; C) All 9 slices are imported into COMSOL for further study.

After all sample slices are imported into COMSOL, triangular and quadrilateral surface elements will be created to connect one slice to its adjacent slices (Figure 5.26A). These connected slices will be then knitted into one complete solid computational domain and meshed using FEM so the 3D boundary conditions from equation (5) and (6) can be applied accordingly (Figure 5.26B). Same concentration which is 1000 ppm of insulin solution is mounted on the top surface of the model and the cumulative concentrations at the bottom surface are recorded for 48h with a 4h interval (Figure 5.26C). The numerical results for passive and MNs enhanced diffusion are shown in Figure 5.27.
Figure 5.26 The process of 3D modelling using sample image slices. A) Two adjacent slices are connected by surface elements; B) All slices are knitted to solid and meshed; C) The diffusion profile when insulin solution is mounted on the top surface of the model.

Figure 5.27 The diffusion profile of the passive diffusion and MNs enhanced diffusion based on the model of Figure 5.26. Insulin concentrations cumulated at the bottom surface are presented.

The cumulative concentrations of passive diffusion in the Figure 5.27 are exactly identical comparing to the 2D results from Figure 5.16 which indicates good consistency of the simulation. However, the concentrations of MNs enhanced diffusion of the 3D model are decreased by approximately 50% due to the size reduction in both horizontal and vertical
directions. We also use this 3D model to test diffusion of lidocaine for 4 hr time duration. The diffusion results of lidocaine in 3D model (Figure 5.28) are showing increment on the time lag which is about 15min longer to reach steady state than the results using 2D model in Figure 5.17.

![Figure 5.28](image)

**Figure 5.28** The diffusion profile of the passive diffusion and MNs enhanced diffusion of lidocaine based on the model of Figure 5.26.

Based on this method, high quality 3D model of MNs treated skin can be achieved in the future if the acquired histological images are consistent and has sufficient quantities. However, this chapter aims to deliver the entire process, thereby experimental results of 3D structured MNs will not be discussed.

**B5 Numerical simulation of effect of different insertion forces**

In chapter 4, we have discussed the effect of different insertion forces on the permeability of large drug molecules such as insulin and BSA. The histological images of the holes created by different MNs under certain forces are also acquired. These images have been imported into the program so the computational domain can be obtained. The theoretical diffusion profile calculated from the program can be used to compare with the experimental data published in our previous paper (Cheung *et al.*, 2014). The histological image of porcine ear skin penetrated by 1100 µm MNs under 69.1N insertion force which is presented in the paper has been chosen as an example image to be imported into the program. The process is shown in Figure 5.29.
Figure 5.29 The histological image of skin penetrated by 1100 µm MNs under 69.1N insertion force is imported into the program: A) the original image from the paper (Cheung et al., 2014); B) the shape of the hole is acquired in MATLAB; C) the diffusion profile is calculated in COMSOL.

Same conditions are configured in COMSOL to maintain the consistency from our previous study. The skin thickness is set to 1.6mm and the total diffusion time is 4 hrs with 1 hr interval. The concentration profile in the receiving compartment from the experiment is compared with the concentration data calculated from the program which are shown in Figure 5.30.

The results from Figure 5.30 are positively presenting the conjunction between the theoretical data can experimental data. It suggests the computational domain deduced from the histological image can well represent the specific condition of the MNs hole caused by certain insertion forces when using 1100 µm MNs.
Figure 5.30 The theoretical data calculated from the program is compared with the experimental data from our previous study. The 1100 µm MNs is applied to the skin under 69.1N insertion force.

5.5 Summary

Numerical simulation based on histological image using an new MATLAB program and COMSOL have been carried out which shows a great potential for accurate numerical modelling of MN enhanced drug delivery. The histological images provide the image of MNs created holes in the skin. This is hypothesised to be more accurate than simply assuming that the MN holes in the skin have the same shape and size as that of the of MNs’ geometry. The histological images indicate an accurate depth of the hole which is required for the numerical simulation. The developed algorithm converts the real images to simulation required coordinates. After importing these data into a numerical simulator (e.g., COMSOL), the diffusion analysis can be easily carried out. The results of the simulation are compared to the experimental data with different insertion forces and have shown high correlation between these two set of results. It is expected that the developed paradigm for numerical simulation of MN based delivery would help the researches to design more efficient MNs systems.
Chapter 6
Conclusions and future works

6.1 Conclusions

This thesis presents new methodologies for the enhancement of the permeability of large molecules in TDD and simulation of the diffusion profiles of drug molecules over time. Two main technologies in the TDD which are MNs and sonophoresis are employed for the experimental study of the enhancement of the permeability. The experimental permeation study is also analysing the effect of insertion force on the enhancement of diffusion. In the theoretical study, a new paradigm of MNs modelling method is also developed in order to obtain higher accuracy for the simulation of the diffusion profiles.

To prove the concept of the enhancement by using ultrasound combined with MNs, various permeation experiments have been conducted. Two commercial solid MNs (1100 µm and 1400 µm length) are used to pretreat the porcine ear skin. After the porous base is prepared, 20 kHz sonophoresis is then employed on the skin samples with different output parameters (power, duration, and duty cycles etc.). The temperature is monitored during the ultrasound administration to ensure the sonophoresis treatment is safe for clinic use and not causing uncomfortable feelings for the patient. The permeation results on bovine serum albumin (>60 kDa) are positive where the best combination (1400 µm MNs combined with 15-W sonophoresis) are indicating that the permeability of BSA are 10 times higher than passive diffusion and 2 times higher than individually applying ultrasound or MNs. This confirms the feasibility of ultrasound combined with MNs as effective and painless method for the enhancement of large molecules.

The insertion depth of the MNs used in the permeation study is also examined. The MNs treated skin sample is stained in methylene blue and then sectioned into 15 µm thick slices in order to expose the cross-sectional view of the hole. The histological images indicate the real insertion depth are less than the length of the MNs whilst factors such as the shape of the hole, the integrity of the SC layer are all varied according to different insertion forces. Therefore, permeation study for the purpose of exploring the relationship between different insertion forces and permeability has been conducted. The ratio between the permeated molecules and the molecules in the donor compartment under 69.1N insertion force for insulin (5.8 kDa) and BSA (66 kDa) are 37.1% and 0.585%, respectively. Although the permeability increment of large molecules in TDD by applying high insertion force is optimistic, but the pain involved during the administration needs to be particularly considered.
Besides, the understanding of quantitative relationship between insertion force and permeability is imperative as it is a constructive factor for all MNs related researches.

The theoretical study of MNs enhanced TDD is also presented in this thesis. Instead of performing simulation on the model of MNs geometry, a new paradigm of MNs modelling is introduced which is based on the histological image of holes created by MNs. This is due to the real insertion depth of 1400 µm MNs is only half of its length according to the histological image. An in-house MATLAB program is developed to capture the outline of the hole from the histological image so the simulation can be conducted on the acquired computational domain. The simulation is employed on both 1 slice (2D) and multi-slices (3D) samples where the concentration profile of insulin are calculated. The correlation between the simulation results and the experimental data are strong whether the MNs is inserted under thumb pressure or higher forces. Hence, the privileges of this new modelling method has been demonstrated not only for the purpose of achieving higher accuracy, but also for extending the utilization of the simulation to adapt different application scenarios, i.e., insertion forces, insertion durations etc. It is complicated when adding all those insertion conditions to the skin deformation model built on the MNs geometry, whereas the new method can directly connect those conditions to the calculated permeation results because all of those conditions are already included in the histological images.

6.2 Future works

The concept of ultrasound combined with MNs for the enhancement of permeability in TDD has been proved as an efficient and feasible method. The specific combination protocol of these two technologies have been introduced in this thesis. However, the potential of this combination method can be further developed in several ways:

(i) The 1100 µm and 1400 µm MNs we use for diffusion experiment in Chapter 3 has adequate length to disrupt the SC layer. However, the contact area where the cavitation can interact with viable epidermis is limited due to the thin structure of this aforementioned MNs. Therefore, MNs that can produce additional contact area is necessary in order to maximize the effect of the micro-jet generated by sonophoresis. This will require customized MNs design which should be based on histological images that can depict the entire contact area exposed by the MNs.

(ii) The localised transport regions mentioned in Chapter 3 indicate that the ultrasound energy is dispersed over an expanded area on the skin comparing to the tip area of the ultrasound transducer. Therefore, the required ultrasound output for cavitation is
unnecessarily increased due to the energy lose. A better idea is to use the focused ultrasound transducer with curved tip surface so the sound field can be then confined in certain area on the skin. This will lead to a great amount of reduction on the ultrasound output energy which is directly related to the size of the ultrasound device.

(iii) As we have proved in Chapter 4, the insertion force applied on the MNs will directly affect the permeation results on different drug molecules. Those results provide important information for the development of ultrasound combined with MNs method. The relationship between the insertion force and the permeation of drug molecules can be further extended to optimize the process when the MNs is occupied to pre-treat the skin. Within a safe range where the administration remains painless, the optimized insertion force can provide additional permeability increment regardless of the geometry of MNs or the ultrasound application.

The simulation results based on the histological images are presenting a very promising future. An example 3D model is constructed by multi-layers of the 2D histological images which reveals the possibility of using this modelling method to reconstruct the entire hole created by MNs. However, the MATLAB program can only provide the computational domain for simulation where the diffusion profile is calculated by FEM software. Therefore, there is a major difficulty when considerable amount of 2D computational domains are imported to the FEM software for reconstruction because the coordinate of the domain have to be imported manually which requires great effort to perform. A sequence function for importing the 2D computational domains into FEM software is imperative. This will requires the cooperation of the company who can add this simple function to automatically read the coordinate of the domains in sequence. Furthermore, the histological images can be correlated to the insertion forces. The simulation results can help us to understand the diffusion profiles of large molecules in the skin when different insertion forces are employed.
Reference


Pankratz A, Forecasting with univariate Box-Jerkins models concepts and cases, New York: John Wiley & Sons, 1983.


Verbaan FJ, Bal SM, van den Berg DJ, Groenink WH, Verpoorten H, Lütgte R, Bouwstra JA, “Assembled microneedle arrays enhance the transport of compounds varying over a large


Appendix: MATLAB Code

Main code

clear all
%create se matrix for dilation accuracy
se=ones(5,5);
%set maximum debris size to 30*30
si=30;
%read microtome image as A
A=imread('09093827.tif');
%convert rgb image A to gray scale image G
G=rgb2gray(A);
%run Canny edge detection on G and acquire binary image bw
[bw,thresh]=edge(G,'canny');
%image dilation on bw using se
di=imdilate(bw,se);

%draw left frame line on di
for x=1:599
    if di(x,1)==1 & di((x+1),1)==0
        di((x+1),1)=1;
    else
        continue
    end
end
%draw right frame line on di
for x=1:599
    if di(x,800)==1 & di((x+1),800)==0
        di((x+1),800)=1;
    else
        continue
    end
end
%draw bottom frame line on di
for y=1:799
    if di(600,y)==1 & di(600,(y+1))==0
        di(600,(y+1))=1;
    else
        continue
    end
end

%fill holes on di
fi=imfill(di,'holes');
%set X as reference image matrix
X=fi;
%set Y as detection and elimination matrix
Y=X;

%run edge detection and debris elimination program on full image
for x=2:599
    for y=2:799
        if Y(x,y)==1 & Y((x+1),(y-1))==0 & Y(x,(y-1))==0 & Y((x-1),(y-1))==0 & Y((x-1),y)==0
            %find every left top positive corners in the image
            %set x1 as reference row coordinate
x1=x;
%set y1 as reference column coordinate
y1=y;
%run first edge detection script
LTP1;
else
    continue
end
end
fi=Y;
%%
%create an empty matrix for the contour of the skin
Z=zeros(600,800);
%acquire contour of the skin image
for y=1:800
    for x=1:600
        if x<600 & fi(x,y)==1 & fi((x+1),y)==0
            Z(x,y)=1;
        elseif x>1 & fi(x,y)==1 & fi((x-1),y)==0
            Z(x,y)=1;
        elseif y >1 & fi(x,y)==1 & fi(x,(y-1))==0
            Z(x,y)=1;
        elseif y<800 & fi(x,y)==1 & fi(x,(y+1))==0
            Z(x,y)=1;
        else
            continue
        end
    end
end
%%
%create new matrix M as organized import matrix for COMSOL
M=[C(1,1),C(1,2)];
%set k as row maker for matrix M
k=1;
%reset tracker l
l=0;
%matrix size counter
m=1;
%empty the first row of matrix C
C(1,:)=[];
%
%organize the matrix C for connected contour
while l<800
    for x=1:r
        if C(x,1)==M(k,1) & abs(C(x,2)-M(k,2))==1
            k=k+1;
            M(k,1)=C(x,1);
            M(k,2)=C(x,2);
            C(x,:)=[];
            l=M(k,1);
            r=r-1;
            m=m+1;
            break
        elseif C(x,2)==M(k,2) & abs(C(x,1)-M(k,1))==1
            k=k+1;
            M(k,1)=C(x,1);
            M(k,2)=C(x,2);
            C(x,:)=[];
            r=r-1;
            m=m+1;
            l=M(k,1);
            break
        elseif abs(C(x,1)-M(k,1))==1 & abs(C(x,2)-M(k,2))==1
            k=k+1;
            M(k,1)=C(x,1);
            M(k,2)=C(x,2);
            C(x,:)=[];
            r=r-1;
            m=m+1;
            l=M(k,1);
            break
        else
            continue
        end
    end
    l=M(k,1);
    r=r-1;
    m=m+1;
end
%
%convert pixels to millimeters
for x=1:m
    M(x,1)=M(x,1)/806250;
    M(x,2)=M(x,2)/806250;
end
%
%add start coordinate and terminal coordinate for COMSOL
M((m+1),1)=M(m,1);
M((m+1),2)=0;
M((m+2),1)=0;
M((m+2),2)=0;

Debris identification scripts

LTP1:
if Y((x-1),(y+1))==1
    if Y((x-2),(y+1))==0
x=x-1;
y=y+1;
if x==1 | y==800 | abs(x-x1)>si | abs(y-y1)>si
    continue
else
    LTP1;
end
else
    x=x-1;
y=y+1;
if x==1 | y==800 | abs(x-x1)>si | abs(y-y1)>si
    continue
else
    RBN3;
end
elseif Y(x,(y+1))==1
    y=y+1;
    if y==800 | abs(x-x1)>si | abs(y-y1)>si
        continue
    else
        LTP1;
    end
elseif Y((x+1),(y+1))==1
    x=x+1;
y=y+1;
    if x==600 | y==800 | abs(x-x1)>si | abs(y-y1)>si
        continue
    else
        LTP1;
    end
else
    RTP2;
end

RTP2:

if Y((x+1),(y+1))==1
    if Y((x+1),(y+2))==0
        x=x+1;
y=y+1;
        if x==600 | y==800 | abs(x-x1)>si | abs(y-y1)>si
            continue
        else
            RTP2;
        end
    else
        x=x+1;
y=y+1;
        if x==600 | y==800 | abs(x-x1)>si | abs(y-y1)>si
            continue
        else
            LBN5;
        end
    end
else
    x=x+1;
y=y+1;
    if x==600 | y==800 | abs(x-x1)>si | abs(y-y1)>si
        continue
    else
        RTP2;
    end
endif
else
    RTP2;
end
elseif Y((x+1),(y-1))==1
    x=x+1;
    y=y-1;
    if x==600 | y==0 | abs(x-x1)>si | abs(y-y1)>si
        continue
    else
        RTP2;
    end
else
    RBP4;
end

RBN3:

if Y((x-1),(y-1))==1
    if Y((x-1),(y-2))==0
        x=x-1;
        y=y-1;
        if x==1 | y==1 | abs(x-x1)>si | abs(y-y1)>si
            continue
        else
            RBN3;
        end
    else
        x=x-1;
        y=y-1;
        if x==1 | y==1 | abs(x-x1)>si | abs(y-y1)>si
            continue
        else
            RTN6;
        end
    end
else
    if x==x1 & y==y1
        LTP1E;
    else
        LTP1;
    end
elseif Y((x-1),(y+1))==1
    if x==x1 & y==y1
        LTP1E;
    else
        LTP1;
    end
else
    if x==x1 & y==y1
        LTP1E;
    else
        LTP1;
    end
end

RBP4:
if Y((x+1),(y-1))==1
    if Y((x+2),(y-1))==0
        x=x+1;
y=y-1;
        if x==600 | y==1 | abs(x-x1)>si | abs(y-y1)>si
            continue
        else
            RBP4;
    end
else
    x=x+1;
y=y-1;
    if x==600 | y==1 | abs(x-x1)>si | abs(y-y1)>si
        continue
    else
        LTN8;
    end
end
elseif Y(x,(y-1))==1
    y=y-1;
    if y==1 | abs(x-x1)>si | abs(y-y1)>si
        continue
    else
        RBP4;
    end
elseif Y((x-1),(y-1))==1
    x=x-1;
y=y-1;
    if x==1 | y==1 | abs(x-x1)>si | abs(y-y1)>si
        continue
    else
        RBP4;
    end
else
    LBP7;
end

LBN5:

if Y((x-1),(y+1))==1
    if Y((x-2),(y+1))==0
        x=x-1;
y=y+1;
        if y==1 | y==800 | abs(x-x1)>si | abs(y-y1)>si
            continue
        else
            LBN5;
        end
else
    x=x-1;
y=y+1;
    if x==1 | y==800 | abs(x-x1)>si | abs(y-y1)>si
        continue
    else
        RBN3;
    end
elseif Y(x,(y+1))==1
    y=y+1;
if y==800 | abs(x-x1)>si | abs(y-y1)>si
    continue
else
    LBN5;
end
elseif Y((x+1),(y+1))==1
    x=x+1;
    y=y+1;
    if x==600 | y==800 | abs(x-x1)>si | abs(y-y1)>si
        continue
    else
        LBN5;
    end
else
    RTP2;
end

RTN6:

if Y((x+1),(y-1))==1
    if Y((x+2),(y-1))==0
        x=x+1;
        y=y-1;
        if x==600 | y==1 | abs(x-x1)>si | abs(y-y1)>si
            continue
        else
            RTN6;
        end
    else
        x=x+1;
        y=y-1;
        if x==600 | y==1 | abs(x-x1)>si | abs(y-y1)>si
            continue
        else
            LTN8;
        end
    end
else
    y=y-1;
    if y==1 | abs(x-x1)>si | abs(y-y1)>si
        continue
    else
        RTN6;
    end
elseif Y(x,(y-1))==1
    y=y-1;
    if y==1 | abs(x-x1)>si | abs(y-y1)>si
        continue
    else
        RTN6;
    end
elseif Y((x-1),(y-1))==1
    x=x-1;
    y=y-1;
    if x==1 | y==1 | abs(x-x1)>si | abs(y-y1)>si
        continue
    else
        RTN6;
    end
else
    LBP7;
end

LBP7:

if Y((x-1),(y-1))==1
    if Y((x-1),(y-2))==0
x=x-1;
y=y-1;
if x==1 | y==1 | abs(x-x1)>si | abs(y-y1)>si
    continue
else
    LBP7;
end
else
    x=x-1;
y=y-1;
if x==1 | y==1 | abs(x-x1)>si | abs(y-y1)>si
    continue
else
    RTN6;
end
elseif Y((x-1),y)==1
    x=x-1;
    if x==1 | abs(x-x1)>si | abs(y-y1)>si
        continue
    else
        LBP7;
    end
elseif Y((x-1),(y+1))==1
    if x==x1 & y==y1
        LTP1E;
    else
        LTP1;
    end
else
    if x==x1 & y==y1
        LTP1E;
    else
        LTP1;
    end
end

LTN8:

if Y((x+1),(y+1))==1
    if Y((x+1),(y+2))==0
        x=x+1;
y=y+1;
        if x==600 | y==800 | abs(x-x1)>si | abs(y-y1)>si
            continue
        else
            LTN8;
        end
    else
        x=x+1;
y=y+1;
        if x==600 | y==800 | abs(x-x1)>si | abs(y-y1)>si
            continue
        else
            LBN5;
        end
    end
else
    x=x+1;
y=y+1;
    if x==600 | y==800 | abs(x-x1)>si | abs(y-y1)>si
        continue
    else
        LTN8;
    end
else
    x=x+1;
y=y+1;
    if x==600 | y==800 | abs(x-x1)>si | abs(y-y1)>si
        continue
    else
        LBN5;
    end
end
elseif Y((x+1),y)==1
    x=x+1;
    if x==600 | abs(x-x1)>si | abs(y-y1)>si
        continue
    else
        LBN5;
    end
else
    x=x+1;
y=y+1;
    if x==600 | y==800 | abs(x-x1)>si | abs(y-y1)>si
        continue
    else
        LTN8;
    end
else
    x=x+1;
y=y+1;
    if x==600 | y==800 | abs(x-x1)>si | abs(y-y1)>si
        continue
    else
        LTN8;
    end
else
    x=x+1;
y=y+1;
    if x==600 | y==800 | abs(x-x1)>si | abs(y-y1)>si
        continue
    else
        LBN5;
    end
end

LTN8:
Debris elimination scripts

LTP1E:

```plaintext
if Y((x-1),(y+1)) == 1
    if Y((x-2),(y+1)) == 0
        Y(x,y) = 0;
        x = x-1;
        y = y+1;
        LTP1E;
    else
        Y(x,y) = 0;
        Y(x,(y+1)) = 0;
        x = x-1;
        y = y+1;
        RBN3E;
    end
else
    Y(x,y) = 0;
    Y(x,(y+1)) = 0;
    x = x-1;
    y = y+1;
    LTP1E;
end
elseif Y(x,(y+1)) == 1
    Y(x,y) = 0;
    y = y+1;
    LTP1E;
elseif Y((x+1),(y+1)) == 1
    Y(x,y) = 0;
    x = x+1;
    y = y+1;
    LTP1E;
elseif Y((x+1),y) == 0 & Y((x+1),(y-1)) == 0
    Y(x,y) = 0;
    continue
else
    RTP2E;
end
```

RTP2E:

```plaintext
if Y((x+1),(y+1)) == 1
    if Y((x+1),(y+2)) == 0
        Y(x,y) = 0;
        x = x+1;
    else
        Y(x,y) = 0;
        y = y+1;
    end
```
```plaintext
y=y+1;
RTP2E;
else
  Y(x,y)=0;
  Y((x+1),y)=0;
x=x+1;
y=y+1;
LBN5E;
end
elseif Y((x+1),y)==1
  Y(x,y)=0;
x=x+1;
RTP2E;
elseif Y((x+1),(y-1))==1
  Y(x,y)=0;
x=x+1;
y=y-1;
RTP2E;
elseif Y(x,(y-1))==0 & Y((x-1),(y-1))==0
  Y(x,y)=0;
  continue
else
  RBP4E;
end
RBN3E:

if Y((x-1),(y-1))==1
  if Y((x-1),(y-2))==0
    Y(x,y)=0;
x=x-1;
y=y-1;
RBN3E;
else
  Y(x,y)=0;
  Y((x-1),y)=0;
x=x-1;
y=y-1;
  RTN6E;
end
elseif Y((x-1),y)==1
  Y(x,y)=0;
x=x-1;
RBN3E;
elseif Y((x-1),(y+1))==1
  LTP1E;
elseif Y(x,(y+1))==0 & Y((x+1),(y+1))==0
  Y(x,y)=0;
  continue
else
  LTP1E;
end
RBP4E:

if Y((x+1),(y-1))==1
  if Y((x+2),(y-1))==0
    Y(x,y)=0;
x=x+1;
y=y-1;
else
  Y(x,y)=0;
  Y((x+1),y)=0;
x=x+1;
y=y+1;
```

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RBF4E;
else
Y(x,y)=0;
Y(x,(y-1))=0;
x=x+1;
y=y-1;
end
elseif Y(x,(y-1))==1
Y(x,y)=0;
y=y-1;
RBP4E;
elseif Y((x-1),(y-1))==1
Y(x,y)=0;
x=x-1;
y=y-1;
RBP4E;
elseif Y((x-1),y)==0 & Y((x-1),(y+1))==0
Y(x,y)=0;
continue
else
LBP7E;
end

RBN5E:

if Y((x-1),(y+1))==1
  if Y((x-2),(y+1))==0
    Y(x,y)=0;
x=x-1;
y=y+1;
LBN5E;
  else
    Y(x,y)=0;
    Y(x,(y+1))=0;
x=x-1;
y=y+1;
    RBN3E;
  end
else Y(x,(y+1))==1
  Y(x,y)=0;
y=y+1;
LBN5E;
elseif Y((x+1),(y+1))==1
  Y(x,y)=0;
x=x+1;
y=y+1;
LBN5E;
elseif Y((x+1),y)==0 & Y((x+1),(y-1))==0
  Y(x,y)=0;
  continue
else
  RTP2E;
end

RTN6E:

if Y((x+1),(y-1))==1
  if Y((x+2),(y-1))==0


\text{LBP7E:}

\begin{verbatim}
    if Y((x-1),(y-1))==1
        if Y((x-1),(y-2))==0
            Y(x,y)=0;
            x=x-1;
            y=y-1;
            LBP7E;
        else
            Y(x,y)=0;
            Y((x-1),y)=0;
            x=x-1;
            y=y-1;
            RTN6E;
    end
elseif Y((x-1),y)==1
    Y(x,y)=0;
    x=x-1;
    LBP7E;
elseif Y((x-1),(y+1))==1
    LTP1E;
elseif Y(x,(y+1))==0 & Y((x+1),(y+1))==0
    Y(x,y)=0;
    continue
else
    LTP1E;
end
\end{verbatim}

\text{LTN8E:}

\begin{verbatim}
    if Y((x+1),(y+1))==1
        if Y((x+1),(y+2))==0
            Y(x,y)=0;
        else
            Y((x+1),(y+1))=0;
            LTP1E;
    end
elseif Y((x+1),(y+1))==1
    LTP1E;
elseif Y(x,(y+1))==0 & Y((x+1),(y+1))==0
    Y(x,y)=0;
    continue
else
    LTP1E;
end
\end{verbatim}
x=x+1;
y=y+1;
LTN8E;
else
    Y(x,y)=0;
    Y((x+1),y)=0;
    x=x+1;
    y=y+1;
    LBN5E;
end
elseif Y((x+1),y)==1
    Y(x,y)=0;
    x=x+1;
    LTN8E;
elseif Y((x+1),(y-1))==1
    Y(x,y)=0;
    x=x+1;
    y=y-1;
    LTN8E;
elseif Y(x,(y-1))==0 & Y((x-1),(y-1))==0
    Y(x,y)=0;
    continue
else
    RBP4E;
end
HPLC calibration and chromatograph examples

**BSA 10-100**

- Formula: \( y = 1.337x \)
- \( R^2 = 0.9635 \)

BSA calibration results from 10 ppm to 100 ppm

**BSA 100-1000**

- Formula: \( y = 2.0269x \)
- \( R^2 = 0.9914 \)

BSA calibration results from 100 ppm to 1000 ppm
Chromatography examples for BSA concentration measurement

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Chromatogram</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 ppm</td>
<td><img src="image1" alt="10 ppm Chromatogram" /></td>
</tr>
<tr>
<td>40 ppm</td>
<td><img src="image2" alt="40 ppm Chromatogram" /></td>
</tr>
<tr>
<td>80 ppm</td>
<td><img src="image3" alt="80 ppm Chromatogram" /></td>
</tr>
<tr>
<td>200 ppm</td>
<td><img src="image4" alt="200 ppm Chromatogram" /></td>
</tr>
<tr>
<td>600 ppm</td>
<td><img src="image5" alt="600 ppm Chromatogram" /></td>
</tr>
<tr>
<td>1000 ppm</td>
<td><img src="image6" alt="1000 ppm Chromatogram" /></td>
</tr>
</tbody>
</table>