A Microscopic Investigation of Transdermal Distribution of Drug Analogues

BAFA501

Bachelor Project

(01.02.2018-01.06.2018)

Author

Katharina Nielsen

kani215@student.sdu.dk

Pharmacy 3th year

Main supervisor

Associate Professor, Ph.D. Jonathan Brewer

brewer@memphys.sdu.dk

Department of Physics, Chemistry and Pharmacy

University of Southern Denmark

Campusvej 55

5230 Odense M, Denmark

Contents

Abstract

The penetration of selected fluorescent drug analogues were investigated on both intact and perforated skin using Franz diffusion cells. Human abdominal skin samples were perforated by microneedles using a dermaroller device (with a needle length of 1.5 mm) at four different forces (10, 20, 30 and 35 N) and a control was also made with an intact stratum corneum to comparison. Two selected drug analogues; fluorescent labeled Dextran (70000 MW) and Green Fluorescent Polymer Microspheres (1.0 µm), were applied to every skin sample and the fluorescens were left to penetrate the skin for 22 hours. The skin samples were cut into cross sections and well-defined microchannels were identified and visualized using Laser Scanning Confocal Microscopy.

Firstly, the depth of the microchannels were investigated in association to the force applied and results indicated that the dermaroller device made substantial microchannels in the stratum corneum. It was, however, observed that not all microchannels had a disrupted stratum corneum. Image analysis showed that no matter what force was applied, it was more common for the dermaroller to make dents rather than to make microchannels with a disrupted stratum corneum. Furthermore, it was observed that there was no clear correlation between the depth of a microchannel and the applied force. However, there was a correlation between force applied to the skin and the how the fluorescens penetrated into the skin.

Secondly, the visualized images were investigated of how Dextran and Green Fluorescent Polymer Microspheres penetrated the skin. Results showed how an increased force lead to a deeper and larger penetration profile of both fluorescens, whilst the lowest force (10 N) did not show a prominent penetration. Therefore, results concluded that a force between 20 N to 35 N should be applied for substantial penetration enhancement.

Dansk resumé

Penetrationen af udvalgte fluorescerende lægemiddel analoger blev undersøgt i både intakt og perforeret hudprøver ved benyttelse af Franz diffusionskamre. Humane abdominal hudprøver blev perforeret med mikronåle ved brug af en dermaruller (med en nåle længde på 1.5 mm) under fire forskellige tryk (10, 20, 30 og 35 N) og ydermere blev der lavet en kontrol med et intakt stratum corneum. To udvalgte lægemiddel analoger, Dextran farvet med fluorescens (70000 MW) og Green Fluorescent Polymer Microsphere (1.5 mm) blev påført til alle hudprøverne og de blev sat til at penetrere huden i 22 timer. Hudprøverne blev skåret i tværsnit og veldefinerede mikrokanaler blev identificeret og visualiseret ved brug af Light Scanning Confocal Microscopy.

Først blev dybden af mikrokanalerne undersøgt og resultaterne indikererede at dermarulleren lavede tydelige mikrokanaler i stratum corneum. Dog blev det observeret at ikke alle mikrokanaler havde et ødelagt stratum corneum. Billede analysen viste at uanset hvilket tryk der blev tilført, så var det mere almindeligt at dermarulleren lavede fordybninger frem for mikrokanaler med et ødelagt stratum corneum. Det blev yderligere observeret at der ikke var nogen klar korrelation mellem påført tryk på huden og hvordan de fluorescerende stoffer penetrerede ned i huden. Der blev dog observeret en klar korrelation mellem det påførte tryk på huden og hvordan fluorescens penetrerede huden.

Dernæst blev de visualiserede billeder undersøgt for hvordan Dextran og Green Fluorescent Polymer Microspheres penetrerede huden. Resultater viste at et jo mere øget trykke, desto dybere en penetration ville forekomme og en større mængde af begge fluorescerende stoffer ville penetrere ned i huden. Derimod viste det laveste tryk (10 N) ikke en betydningsfuld penetration.

Abbreviation list

Word	Abbreviation
Intravenous infusion	IV
Stratum corneum	SC
Phosphate buffered saline	PBS
Laser Scanning Confocal Microscopy	LSCM
Green Fluorescent Polystyrene Microspheres	GFPM
Poly(D,L-lacic-co-glycolic-acid)	PLGA

1. Introduction

The skin is the human body's largest organ, with a broad range of functions. It is widely known for its ability to protect the deeper organs against the outside environment, as the skin creates a robust barrier [3]. From a pharmacological perspective, the skin can be used for topical or continuous systemic administration of therapeutics. Topical delivery is useful when the skin is the main target for treatment. The skin can also be used as a transdermal pathway for systemic delivery, where drugs with short half-lives can be distributed continuously into the systemic circulation. This gives transdermal administration similar properties to intravenous infusion (IV), however transdermal administration is noninvasive, and hospitalization is not required. Like IV, the drug will not become metabolized right away as it bypasses hepatic circulation [1].

Nanoparticles have been widely studied concerning if they can be applied for parental and oral administration. However, more recent studies also focus on whether nanoparticles can be applied for topical drug administration too. Research has indicated that nanoparticles can be used for gradual drug release on the skin surface and maintain the drug concentration in the skin over a longer period. However, it was found that most nanoparticles were unable to penetrate further down than to the stratum corneum (SC) and only a few nanoparticles were able to passively permeate into the skin through hair follicles [9].

Newer research has studied how disrupting SC, the outermost layer of the skin, may improve delivery of therapeutics, for both local delivery but also systemic delivery. This can be preferable since unlike IV, the delivery is non-invasive and can be performed at home. [1]. Several ways to enhance penetration has been studied, such as chemical/lipid enhancers, iontophoresis and heat [5,6]. Although the techniques work differently, they all have the same common goal of disrupting SC by creating microchannels for molecules to pass through. One way of temporarily disrupting the skin is through microneedles, which creates perforated microchannels in the skin. The skin is self-generating, and SC will only be temporarily disrupted, however this still allows a greater number of therapeutics to be delivered through transdermal administration. Still, the movement of nanoparticles through microchannels is a complex process and poorly understood method, that still needs further investigation [6].

The main objective of this project was to apply Laser Scanning Confocal Microscopy for investigation of how two different fluorescent labeled drug analogues penetrated perforated skin treated with a dermaroller Device, with a specific focus on the correlation between the force applied when using the dermaroller and the penetration through the skin.

2. Background and theory

2.1. The skin barrier

The skin barrier is a complex organ with a broad range of diverse functions. Crucial functions include protecting the body against the outside environment, regulating the body temperature and protection against rays from the sun [2]. The structure of the skin consists of a series of layers, that overall form a barrier. The skin is divided into three layers: The outer layer, *epidermis,* a middle layer, *dermis,* and an inner layer, *hypodermis* (see figure 1).

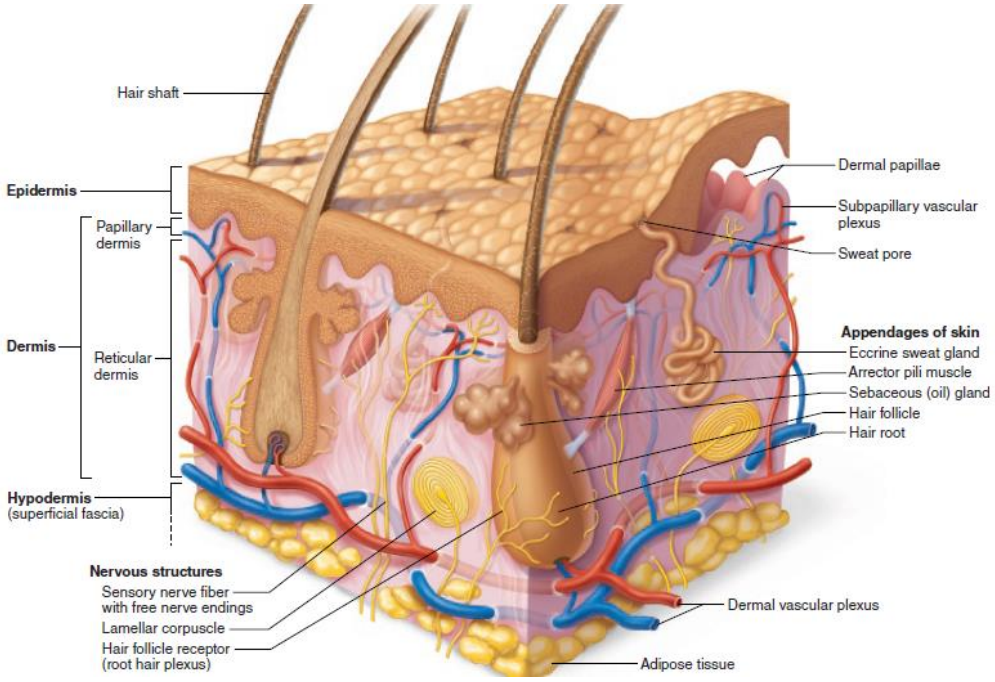


Figure 1: The general structure of the human skin consists of three skin layers: Epidermis, dermis and hypodermis. Picture is reprinted from Pearson Education, Inc. New York, New York (from[4]).

The epidermis consists of four types of cells: *keratinocytes, melanocytes, tactile epithetlial cells, and dendrites cells*, where the keratinocyte is the most common cell with the function of producing keratin, a fibrous protein, giving the epidermis its protective properties. There are four layers to the epidermis: *Stratum corneum (SC), Stratum granulosum, Stratum spinosum and Stratum basale.*

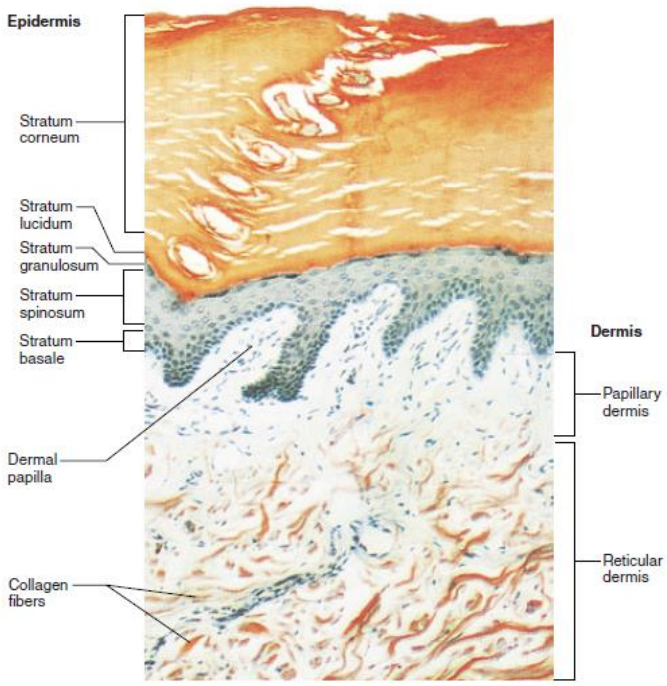


Figure 2: The layers of, epidermis, is divided into four sublayers: Stratum corneum, stratum granulosum, stratum spinosum and stratum basale. Picture is reprinted from Pearson Education, Inc. New York, New York [3].

SC is the outer layer of the skin and functions as a barrier to foreign molecules. This includes potential drugs, and drug delivery across the skin barrier is thereby limited [3]. SC is 10-20 µm thick and consists of approximately 20-30 cell layers, that are densely packed. The layers have dead keratinocytes, filled with keratin because of disintegration of nuclei and organelles due to cell death. [4, 5]

Stratum granulosum consists of 1 to 5 layers of keratinocytes which contain not only pre-keratin intermediate filaments but also keratohyalin granules and lamellar granules. These contribute to SC because keratohyalin help form keratin, and lamellar granules contains hydrophilic glycolipids which are secreted into the extra cellular space. There are glycolipids along the tight junctions between adjacent cells, which prevent major water loss across the epidermis and make the plasma membrane of the cell more resistant to destruction. *Stratum spinosum* is several layers thick and is a source to mitosis. Again, this layer contains keratocytes, but amongst them are dendritic cells that are a part of the immune system. *Stratum basale* is the deepest epidermal layer and is firmly connected to the underlying skin-layer, dermis. It consists of mostly stem cells which will become keratinocytes. (See figure 2)

The second skin layer, the *dermis,* consists of strong, flexible connective tissues and is made up of fibrolast and immune cells. If has two regions: the *papilla dermis* and the *reticular dermis.* The dermis is supplied with nerve fibers, blood vessels, glands and hair follicles (see figure 1). Lastly, dermis is connected to the third skin layer, the hypodermis, which consists of both areolar and adipose connective tissue [4].

2.2. Topical and transdermal administration of drug molecules

The skin barrier is also used as a possible delivery route for therapeutics, which can be classified as either topical and transdermal delivery:

Topical drug delivery is widely used for local disorders on the skin. Here, the drug molecules are presented to the skin surface, where they travel through the SC. There are three main routes: They can either pass through the shunt route using the glans or sebum of the skin, and from there diffuse into the viable epidermis. Another more common route, known as the intracellular route, is used for drugs that can diffuse through the corneocytes and into the viable epidermis. Lastly, there is the intercellular route, where drug molecules partition into the lipid bilayers between corneocytes. From there, they can partition into the viable epidermis (see figure 3).

From there, transdermal delivery of drug molecules can occur if they diffuse to the lower part of the epidermis and travel to the capillaries through the epidermal-dermal junction. However, during transport, the drug may bind to skin components such as keratin or be metabolized by metabolically active enzymes that are present in the skin [3].

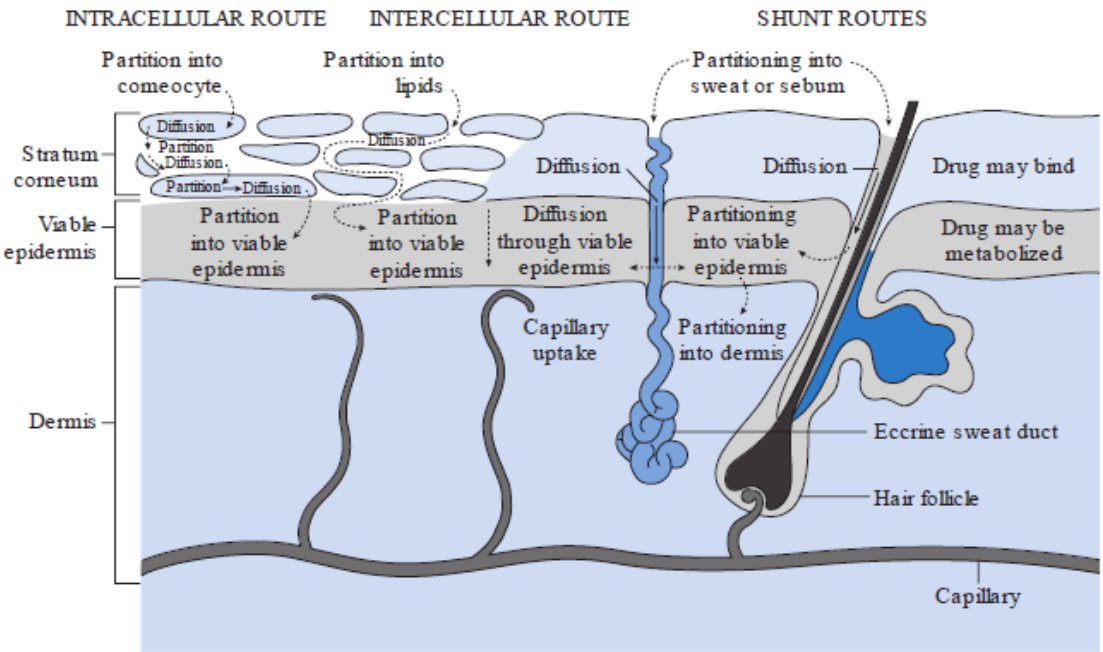


Figure 3: Transdermal drug delivery can happen through the intracellular route, the intercellular route or the shunt routes. Picture is reprinted from Churchill Livingstone Elsevier 2013 (from [3]).

Topical delivery works locally on the skin, whereas transdermal delivery reaches the systemic circulation, and hereby bypasses the liver and avoids undergoing first pass metabolism. This can be a huge advantage compared to oral administration, which may lead to side effects caused by first pass metabolism. However, only certain molecules with specific physiochemical properties can permeate through the skin, and research has proven that nanoparticles are usually primarily restricted the upper layers of epidermis [6]. Furthermore, drug permeation may only gradually be absorbed into the systemic circulation, making the concentration of drug absorbed unpredictable. Enhancement methods for transdermal drug delivery are therefore highly researched [3,9].

2.3. Optimization strategies for more effective penetration of drug molecules across the skin barrier

An extensive problem with IV and subcutaneous administration is patient compliance. Therefore, the field of transdermal delivery is researched extensively with the sole purpose of enabling delivery through the skin with penetration. There are many ways to enhance penetration, such as chemical/lipid enhancers, iontophoresis and heat [5,6]. These techniques all work in differently, but they have the same common goal of disrupting SC by creating microchannels for molecules to pass through.

One of the more commonly investigated methods is microneedling, a method using micron sized needles to create microchannels in the SC. The procedure is easy to perform and relatively painless, making it an alternative candidate to IV [7,10].

Commercially, Dermarollers are used to regenerate skin cells through puncturing the SC. This stimulate production of collagen and elastin, which can treat scarring and wrinkles, and overall improve the skin appearance. The process is easy and leaves little irritation to the skin [11,12]. Dermarollers can be made from stainless steel and consist of a handle attached to a cylinder with microneedles of a certain size (see figure 4).

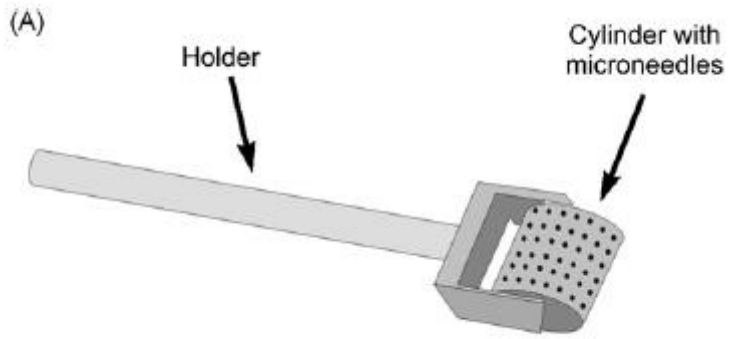


Figure 4: Presentation of a dermaroller device, used to preform skin perforation (from [10]).

The needle length ranges from 0.25-1.5 mm, and the needle length determine the function of the dermarollers' when used in association to cosmetic procedures. The device perforates the SC and creates a limited number of microchannels in the epidermis. Topically applied molecules can more easily travel through the skin layers and access the systemic circulation when they are applied to skin with a disrupted SC [10,13]. Thereby, limitations such as the size of particles can be overcome without causing any irreversible damage to the skin or hurting the patient [14].

2.4. Optimization strategies of nanoparticles

The physiochemical properties of the skin control the transport of molecules across the skin barrier. Such properties include a size less than 500 Da, the molecule is uncharged, and it has a Log P between 1-4, although more lipophilic drugs such as fentanyl can be efficiently delivered [3,13].

Nanoparticles are widely studied in relation to oral and parenteral administration, but lately, they have also been investigated for topical drug administration. Nanoparticles can have positive effects such as supporting the skin with a certain drug over a prolonged time or maintaining a desired drug concentration in the skin. However, research has found that nanoparticles have trouble being delivered to the deeper skin layers and they stay on the skin surface. Studies has since suggested that treating the skin with microneedles prior to application of nanoparticles may improve penetration [9]. Though microchannels created by microneedles should successfully facilitate transdermal penetration of nanoparticle formulations, the movement across the skin barrier is complex and only a limited number of studies are available.

Some impediments have been identified though for transdermal delivery of nanoparticles in formulations, including

i. Non-covalent interaction with the skin surface and/or other components of the tissue
ii. Unstable formulations that may led to phenomenon such as flocculation
iii. Steric hindrance
iv. Degradation of the formulation in the biological environment. [8]

Research has also suggested that smaller nanoparticles can more easily penetrate through both epidermis and dermis compared to larger nanoparticles. This indicates that penetration through microchannels may be a size-dependent and reducing particle size may be beneficial for penetration. Furthermore, research suggests that the permeation rate increases with the concentration of nanoparticles, although only to a certain extent, before the maximal penetration is achieved [9].

Another crucial factor is the surface charge called the zeta potential. It is described as the charge that may develop at the interface between a solid surface and its liquid medium. The zeta potential is a useful tool to understand the stability of pharmaceutic formulations such as suspensions and emulsions. Most colloidal dispersions in aqueous media carry an electric charge, which affects the distribution of ions on surface of the formulation. The zeta potential of colloidal dispersions can be measured using the micro-electrophoresis technique [15,16].

2.5. In vitro experiments testing the penetration of fluorescent particles

This study examines the penetration of fluorescent nanoparticles on human skin treated with a dermaroller device. To perform this, Franz diffusion cells were used. These cells are commonly used for permeation studies but can be used for penetration studies as well. The Franz diffusion cell is made up of two glass compartments: An upper chamber known as the donor compartment, and a lower chamber known as the receptor compartment (see figure 5).

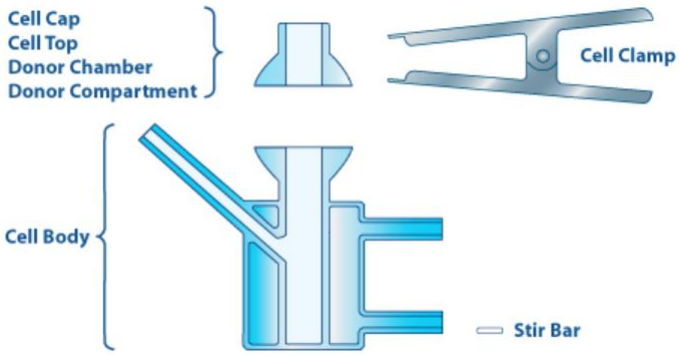


Figure 5: An illustration of a standard Franz cell, consisting of a donor- and receptor compartment (from [17])

Samples can be studied by placing it between the two chambers and securing with a cell clamp. Formulations of interest can be added to the donor compartment and left to permeate and penetrate the samples for a predefined time interval. Fluid is applied to the receptor chamber and can be constantly stirred and held at a constant temperature regarding to optimal conditions for the given experiment [17].

Analytical methods such as microscopy can be applied to investigate the quantity of a formulation that has penetrated the skin. A well-known technique is Laser Scanning Confocal Microscopy (LSCM). It is widely used in biological and biomedical investigations that need imaging of thin optical sections of samples. LSCM is often capable of revealing the presence of just a single molecule and is therefore an optimal tool for investigation of living cells and tissues.

The principle behind LSCM is coherent light is emitted by a laser system through a pinhole aperture. The light hits a dichromatic mirror and will be reflected to hit the specimen at a defined focal plane. Light emitted from the specimen coming from the focal, will pass through the dichromatic mirror and will be focused to a point at the detector pinhole aperture and pass through the aperture to the detector (see figure 6).

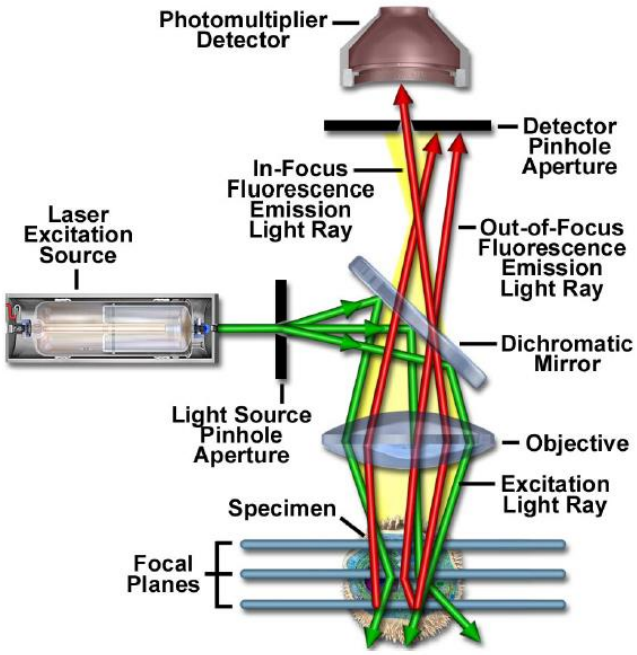


Figure 6: A schematic presentation of Light scanning confocal microscopy (from [19]).

Light emitted from the sample from out of focus plains will not be focused at the pinhole and will be rejected by the pinhole. Light that do not pass through the pinhole is excluded from detection. To observe a different plain in the sample, the objective of LSCM can be moved, so the focus is moved to a new plain of interest. To obtain microscopic images LSCM is connected to a computer with a suitable software, such as LASX.

The technique relies on fluorescence as a way of studying dye binding in fixed cells and living tissue [9]. Fluorospheres™ are described according to their absorption and fluorescence properties, including wavelengths of maximum absorbance and emission, spectral profiles and the fluorescence intensity of the emitted light [18,19].

This experiment uses two types of fluorophores, with the first being Dextran, which are hydrophilic polysaccharides labelled with Texas Red®. It has an excitation maximum of 595 nm and an emission maximum of 615 nm (see figure 7A) [20]. The second is Flourospeheres™ Polystyrene Microspheres, with a yellow-green fluorescence with a size of 1.0 µm. They are formed from polymers such as polystyrene and the particles are spherical. The excitation and emission spectrums are respectively 505 and 515 nm (See figure 7B) [21].

A:

B:

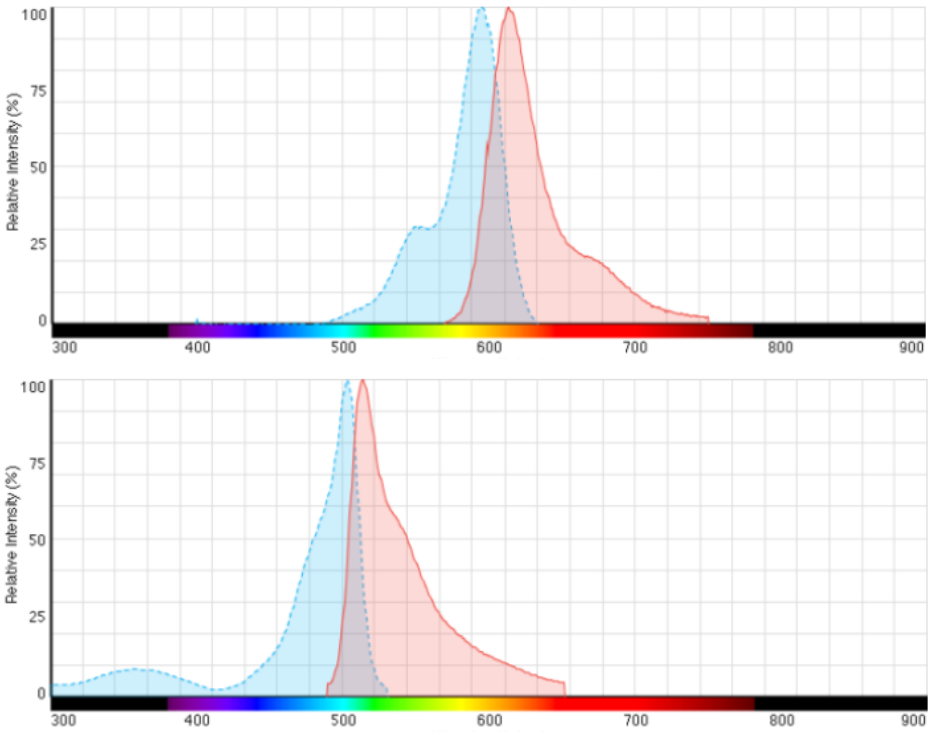


Figure 7A and 7B: (A) Fluorescent spectra of Dextran, Texas Red™ (excitation/emission is 595/61nm 5) and (B) fluorescent spectra of yellow-green fluorescent (excitation/emission is 505/515 nm)

3. Material and methods

3.1. Materials:

Two types of fluorescent drug analogues were used to conduct this experiment: Dextran (70,000 MW, neutral) and 1.0 µm Green Fluorescent polymer Microspheres (GFPM) (1% Solids). Both types were purchased from Thermo Fisher Scientific (see table 1).

Table 1: Schematic information regarding Dextran and Green Fluorescent polymer Microspheres.

Material	Fluorescens	Excitation/emission (nm)	Catalogue number
Dextran	Texas Red®	595/615	D1830
Green Fluorescing polymer microspheres	Duke Scientific Corp.	468/508	G0100

Milli-Q® water, was produced and purified by Milli-Q® Integral Water Purification System for Ultrapure Water and obtained from Millipore, Germany. Phosphate buffered saline (PBS) tablet were obtained from Sigma.

3.2. Preparation of buffer solution

A solution of PBS was prepared by dissolving two tablets in 400 mL of Milli-Q® water, giving a solution with a concentration of 0.01 M phosphate buffer (0,0027 M potassium chloride and 0,137 M sodium chloride) with a pH of 7.4 at 25 °C.

3.3. Preparation of skin samples

Abdominal human skin was obtained from a woman, (age 48, delivered from Odense University Hospital). The skin was prepared by initially removing the fat tissue and thereafter washing the skin with gauze on each side of the sample. Hereafter, the skin was wrapped in tin foil and stored in a freezer (-80 °C) until use. During the experiment, the skin samples were treated ethically, and the use was approved by the Regional Research Ethics Committee of Southern Denmark which were adherent to the Declaration of Helsinki Principles (2008). The skin samples were obtained as unwanted tissue from surgery, for which patient consent was not compulsory. The frozen piece of skin was withdrawn from the freezer and cut into five equal samples with a scalpel and a scissor. Each sample was

approximately 4x4 cm to make sure the skin pieces would fit around the head of the receptor compartment on the Franz diffusion cells. The samples were then kept untreated until they were completely thawed.

Four of the five skin pieces were individually placed on a polystyrene stand and stretched out by four injection needles, placed in each corner of the square piece (see figure 8). To prevent the skin sample from sliding on the polystyrene stand, a layer of foil was placed over the polystyrene base along with a piece of filter paper (not shown).

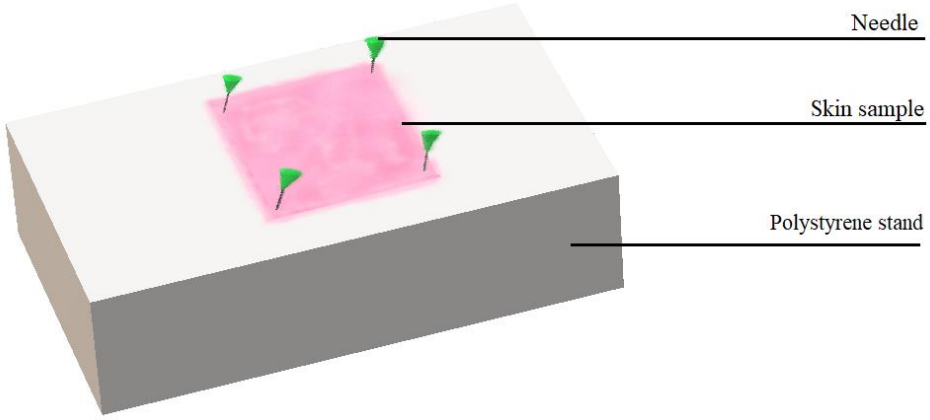


Figure 8: Experimental setup consisting of a polystyrene stand wrapped in tin foil and a piece of filter paper on top (not shown in figure). A skin sample is placed on the stand and needles were inserted at every corner of the sample, so secure it from sliding across the stand.

This experiment tested the penetration of four different forces: 1, 2, 3 and 3.5 kg (hereafter acknowledged in newton by applying Newton's Second Law, F=mg, with F being the force, m being the mass in kg and g being the gravitational constant – approximated to 10 ms^{-2} in this study). Furthermore, there was a control with intact SC. The stretched skin sample was perforated by a dermaroller (DRS DermaRoller), by rolling the device across the skin sample three times with a given force. The dermaroller consisted of 540 titanium needles of length 1.5 mm according to the supplier information. To control which force was applied, the polystyrene stand stood on a household weight, that would report the applied force. The dermaroller was handheld and force was applied by strength, therefore, the force was not consistent through treatment. Four different samples with forces 10, 20, 30 and 35 N were created. One skin sample was left untreated and used as control.

3.4. Penetration studies

Solutions of Dextran and GFPM were prepared for further investigation of the skin samples (see table 2).

Table 2: The fluorescens compounds Dextran and GFPM were prepared in a solution with concentrations of respectively 1 mg/ml and 0.1 mg/ml.

Fluorescent samples used for penetration studies		
Compound	**Concentration (mg/ml)**	**Volume parts**
Dextran	1 mg/ml	0.4 ml dextran to 3.6 ml PBS buffer
1,0 µm green fluorescent polystyrene microspheres	0.1 mg/ml	0.02 ml green fluorescent beads to 3.98 ml PBS buffer

Franz diffusion cells were used to test the penetration of the two types of fluorescent particles. The skin samples were placed on Franz diffusion cells (Permegear Inc., Riegelsville, PA, diffusional area of 3.14 cm^2 and receptor volume of 15 ml). The receptor compartments were filled with PBS buffer, retained at 32 °C ± 1 °C, which is considered the temperature of human skin [22]. Magnetic stirring kept the PBS buffer rotating at consistent speed (500 rpm, RO 10 IKA®). The skin sample was placed with the SC facing the donor compartment on the Franz cell and clamped between the donor and acceptor compartment. 200 µl of Dextran and 100 µl of GFPM were added to each donor cell. The compartments were covered with aluminum foil to prevent the skin and fluorescent particles from drying out. The samples were left for 22 hours on the Franz cell to allow the fluorescens to truly penetrate the skin.

To study the penetration of the fluorescent drug analogues, the experiment was stopped after 22 hours, and the skin samples were removed from Franz diffusion cells. Every skin sample was sliced into 4 equal pieces (approximately 1x1 cm). the samples were immediately frozen using a polystyrene box filled with liquid nitrogen. A bowl filled with 2-methylbutane (produced by Honeywell®) was placed into the polystyrene box. A 1x1 cm skin sample was placed in a 1.5x2.5 cm aluminum box and thereafter inserted into the 2-methylbutane for approximately 45 seconds, then withdrawn and placed in another polystyrene box containing dry ice until further use. For microscopic investigations, the skin samples were attached with O.C.T compound (VWR Chemicals) onto a cryotome holder and sliced in a 45° angle to the machine. The slices were each 25 µm and they were sliced using Cryotome FSE (Thermo Fisher Scientific) operated at -21 °C.

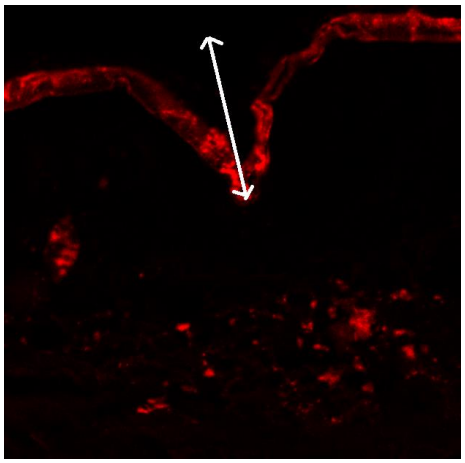
3.5. Confocal microscopy

The skin samples were visualized using a Leica SP8 (Leica Systems, Manheim, Germany) and a suitable software, Leica Application Suite X (LAS X, version 3.1.5.16308). The cross sections of the skin samples were visualized with a 10x/0.75 dry objective. An excitation/emission range of 595/615 nm (for Dextran) and 468/508 nm (for GFPM) was used. The white light laser was turned on for wavelengths where light was excited, and the emission range was chosen to fit each type of fluorescence. The laser power was adjusted to fit the fluorescens, and 36 % was applied for measurements of Dextran samples, while 11.4 % was applied for GFPM. The format of each image was 1024 x 1024 pixels and a zoom of 1.25x was used.

3.6. Image analysis

The images obtained from LSCM were investigated using the program ImageJ (Image processing and Analysis in Java, version 1.52b). The area of the hole was measured using the tool function 'Measure', while depth and width of each microchannel were investigated using the setting 'Straight' in the toolbar. The depth of each hole was measured from the top of the SC down to the bottom of the microchannel (see figure 9A). The width of each microchannel was measured using the setting 'straight' and measuring the middle of the hole, from the outer SC on one side to the outer SC on the other side (see figure 9B).

A



B

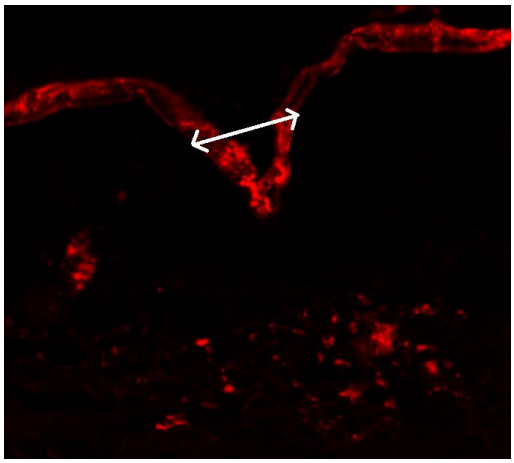
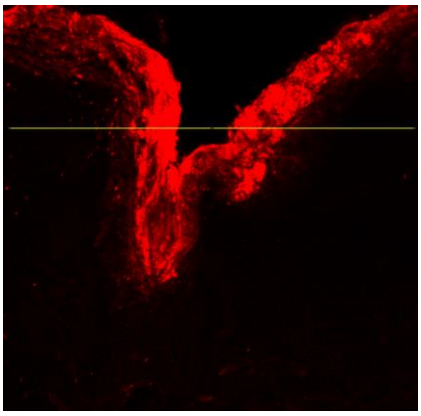


Figure 9A and 9B: Illustration of how the depth (A) and width (B) of each microchannel was measured using ImageJ's tool 'straight'.

To investigate how Dextran penetrate the skin, a custom macro-tool was used to calculate the average intensity of a predefined area of each sample analyzed. The microchannel in every image was defined first and the average intensity could be measured using a predefined area with a width of 2 µm. The intensity shows how much Dextran is present in the skin sample and thereby how it has penetrated. The analysis started at the top of the SC and was set to take 200 measurements, starting from the top of SC and ending 400 µm below (see figure 10A).

A



B

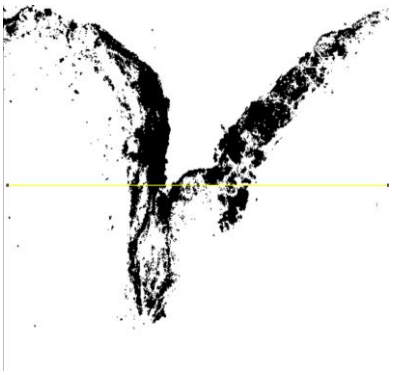


Figure 10A and 10B: (10A) The intensity of Dextran is measured using a custom macro-tool. A predefined area with a width of 2 µm (yellow line) measured from the top of SC and 400 µm down into the cross section. (B) Images obtained of GFPM penetration was measured using another custom macro-tool. The images containing GFPM was first threshold and then the total area of particles was measured by a predefined area of 2 µm (yellow line).

Images obtained with GFPM were analyzed using another custom-made macro tool, that calculated the total areas of particles in a predefined area with a width of 2 µm. This identified the total number of particles that had penetrated into the epidermis as it is proportional to the number of nanoparticles present in the epidermis. To conduct this analysis, each picture with GFPM was treated with threshold to identify the particles (see figure 10B). 200 measurements were obtained, with the 2 µm box starting at the top of SC and ending 400 µm below.

The movement of Dextran horizontally into the epidermis was investigated using the tool ‘straight’, placed from SC into epidermis. The line was 100 µm and the function ‘plot profile’ measured the intensity (see figure 11).

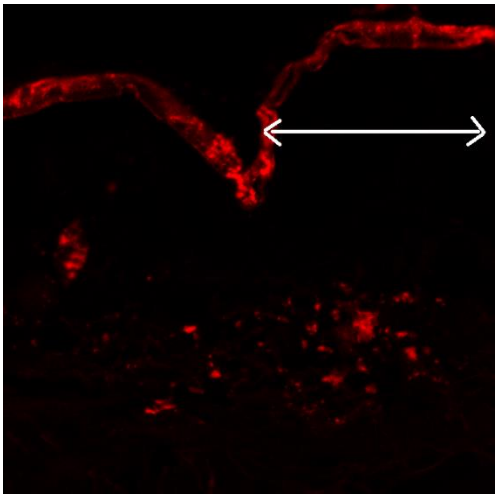
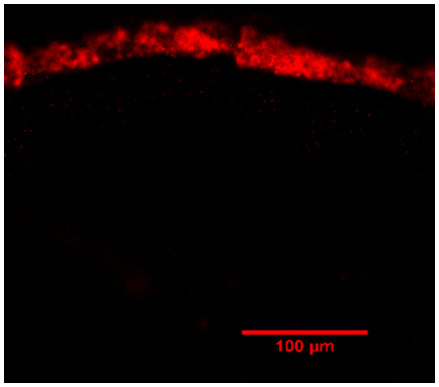
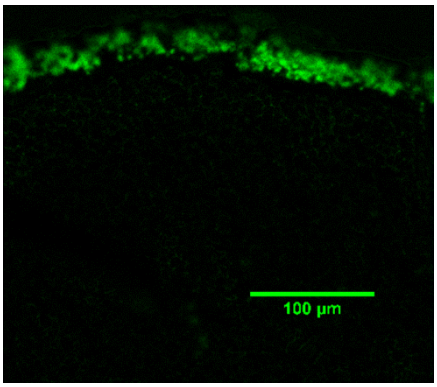
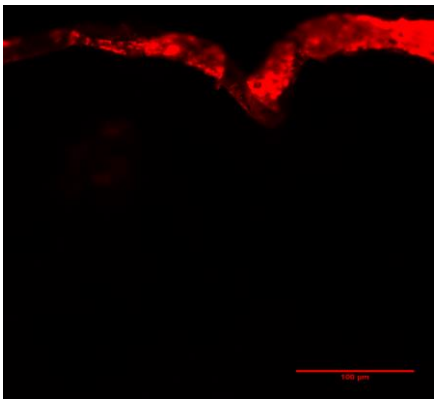
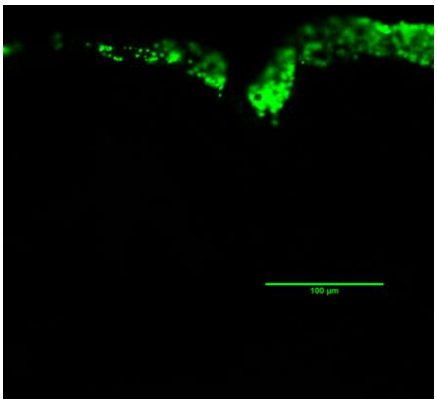


Figure 11: Images featuring Dextran were analysed for Dextran’s movement horizontally into epidermis, using ImageJ’s tool ’straight’.

4. Results

4.1. Visualization of the penetration patterns of Dextran and GFPM

To investigate the penetration of Dextran and GFPM, pictures were taken using LSCM. To visualize cross sections of skin perforated and treated with Dextran, a 1024x1024 format was applied along with a laser intensity of 36%. For Green fluorescent beads, the same format was applied but instead the laser intensity was 11.4%. Both sets of pictures were taken with a zoom of 1.25x. Two independent sets of experiments were investigated. 10 pictures for every force category of each experiment was obtained so overall 20 pictures from every type of force/control was taken (see figure 12).

Force (N)	**Dextran**	Force **(N)**	Green Fluorescent Polymer Microspheres
Control		Control	
10 N		10N	

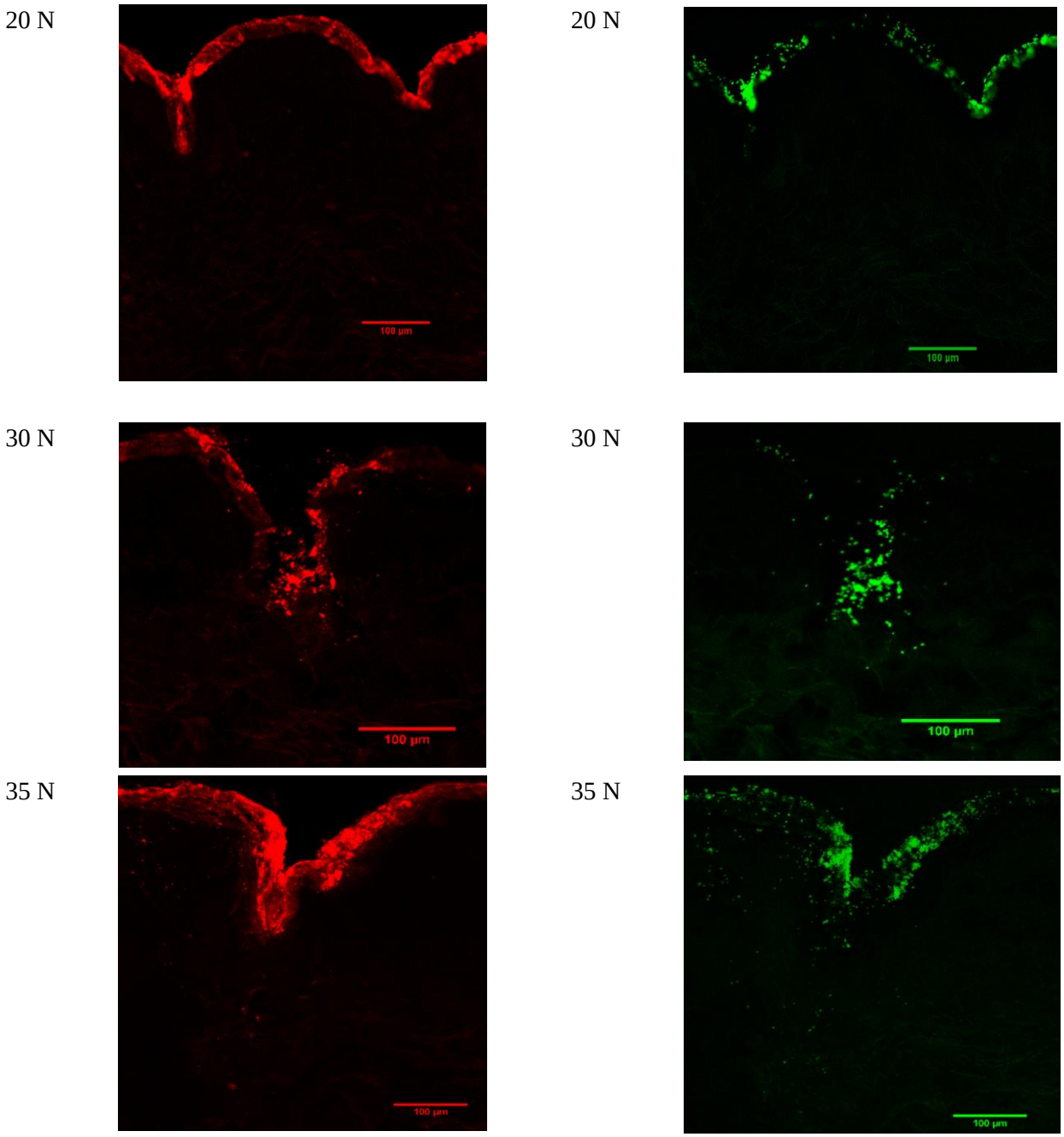


Figure 12: Cross sections of intact and perforated skin samples is displayed. Images to the left demonstrate how Dextran penetrate through the skin at sets of force: 10, 20, 30 and 35 N. Images on the right demonstrate how GFPM penetrate through the skin at sets of force: 10, 20, 30 and 25 N. Pictures were obtained with a 3476 x 3476 format.

The images visualized how SC was affected by the dermaroller. The images obtained of the control shows an intact SC, while pictures obtained of SC treated with the dermaroller shows incisions. Furthermore, the images collected showed how both Dextran and GFPM were more likely to penetrate the viable epidermis on skin samples treated with the dermaroller. The results also showed no distinct evidence that the dermaroller has perforated completely through SC. The images mostly showed dents. However, a limited number of pictures obtained exhibits microchannels that has disrupted the SC (see figure 12C-F).

4.2. Investigation of the depth of microchannels

The depth of a microchannel produced by applying a defined force with the dermaroller device was investigated using LSCM. The samples were divided and analyzed according to the force they were treated with. Every skin sample was perforated three times with the dermaroller device. The force was applied manually and not by machine, which resulted in derivations from the selected force (see Appendix I, Table A1).

The mean depths were calculated using the individual measurements obtained from every set of force. Unfortunately, the same number of measurements of depth were not obtained for all tests. Therefore, the mean depth is calculated from a smaller set of samples in some instances. These have been detailed in table 3 (see Appendix I, Table A2 for measured depth of each microchannel).

Table 3: The mean depth ± standard derivation of microchannels

Force applied (N)	**Mean depth (µm)**	**Number of measurements obtained**
10 N	111.19 µm ± 28.05	19
20 N	104.78 µm ± 30.58	25
30 N	125.52 µm ± 53.22	21
35 N	113.27 µm ± 53.66	14

The results show no distinct relation between the force applied to the skin samples and the mean depth of the microchannels. The results show how a force of 10 N only results in a mean depth of 2.08 µm lesser than 35 N. However, the results also show a significant standard deviation for all the values, particularly 30 N and 35 N, which have standard derivation greater than 50 µm. This indicate that the depth of microchannel can easily have various sizes even though the same force is applied. Furthermore, the images obtained suggest that the dermaroller more commonly made dents rather than microchannels with a disrupted SC (see Appendix 1, figure A1). This phenomenon occurred regardless of what force was applied.

4.3. Analysis of Dextran and Green Fluorescent Polymer Microspheres above the microchannels

An important aspect of this study is examination of how different fluorescent drug analogues penetrate the skin after treatment with a dermaroller. The skin was therefore initially treated with a dermaroller at different forces, and subsequently Dextran and GFPM applied to the skin samples. The sets of skin samples were left for 22 hours to allow the particles to penetrate. LSCM was used to visualize the cross sections of samples using method described on figure 10A and 10B in section 3.6.

Data from the control and every set of force is represented in their individual set of bars. The cross sections were measured from SC and down 400 µm into the skin sample (however only values up to 355 µm is represented). The measured intensity values were divided into approximately 20 µm intervals for every set and the mean values for each interval was calculated (see Appendix II, Figure A2). Selected intervals of the mean values for every set of force is represented in the bar chart (see Figure 13).

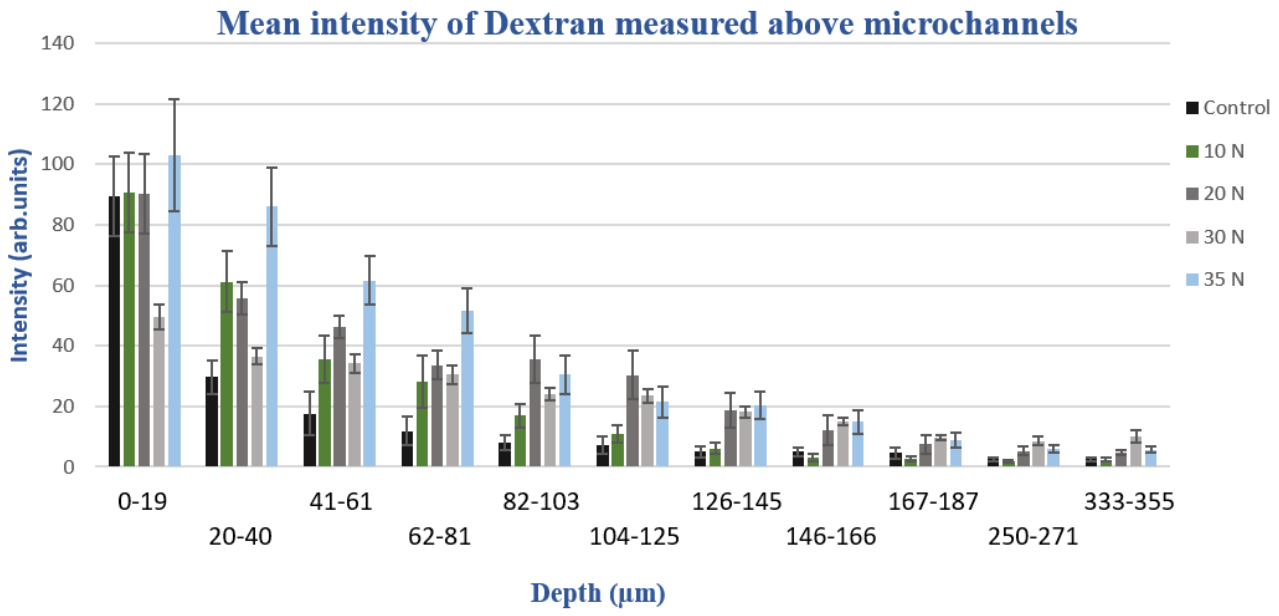


Figure 13: A bar chart presenting the mean values and standard derivations of intensity as a function of the depth (µm) for Dextran.

From figure 13 it is observed how the penetration of Dextran behaves in the skin. It is observed that the control has very little penetration below 40 µm into the skin. As the SC is 10-20 µm thick, this is an indication that Dextran is not capable of penetrating much further than through SC. It was also observed how the perforated samples had increased penetration at a depth below 250 µm compared to the control. Furthermore, there seems to be a clear trend that increased force lead to a deeper penetration and more Dextran can penetrate the skin. This is seen from the force set, 35 N, that has the highest penetration until below approximately 80 µm, and thereafter, still show penetrating qualities until below 167 µm. Lastly, it is also observed how 10 N does not seem to have a prominent penetration below 82 µm, which indicates that a force of 20 N or higher was needed for substantial penetration enhancement.

The same method were performed on images with GFPM. The data set was obtained using method described in figure 10B, section 3.6. A graph was created in the same way as for Dextran, using the highest valuable for number of particles as a starting point. The vertical axis present number of particles as a unit as a function of the depth in µm. This is made possible by multiplying every dataset with the formula for a circle (πr^2), with r being 0.5 µm. the results are presented as a graph, with the highest number of particles as starting point (see figure 14).

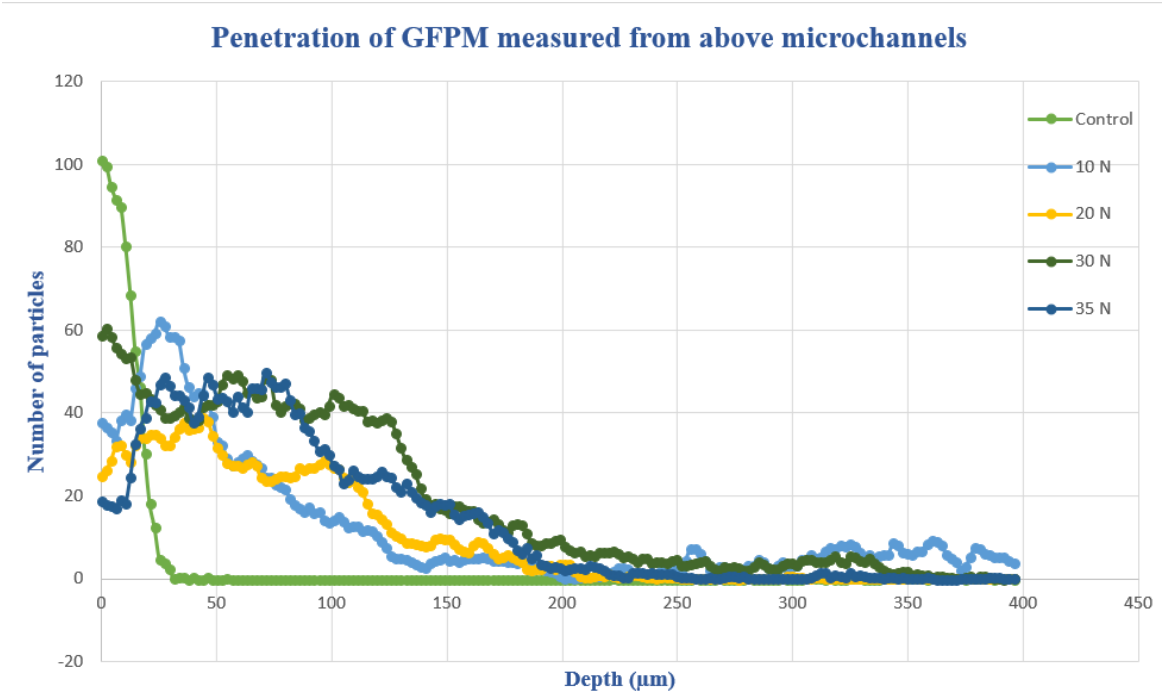


Figure 14: A graph showing the results of GFPM penetration profile in human skin, with number of particles as a function of depth in µm.

Figure 14 demonstrate that all four sets of forces have GFPM present in the epidermis all the way to approximately 120 µm, and 30 and 35 N are observed to have particles present at a depth of 175 µm. For 10 N a fluctuation from around 250-400 µm is observed, which indicates the presence of autofluorescence.

4.4. Analysis of Dextran and GFPM under microchannels

A further visualization of the behavior of Dextran was executed. Images were obtained using the method described in figure 10A, section 3.6., however, the 2 µm box had a starting point for measurement at the end of each microchannel. The data obtained for every forc was normalizeed to analyze the relation between the different forces and penetration. By normalizing the data, it is considered that some images may contain a larger quantity of Dextran. All data was normalized by finding the maximum value for intensity for every single measurement and dividing the rest of the intensity values (using the formula: $Intensity_{normalized} = \frac{intensity}{intensity_{max}}$). Subsequently, the average values were calculated for every set of forces, and the results were inserted into a bar chart (see figure 15).

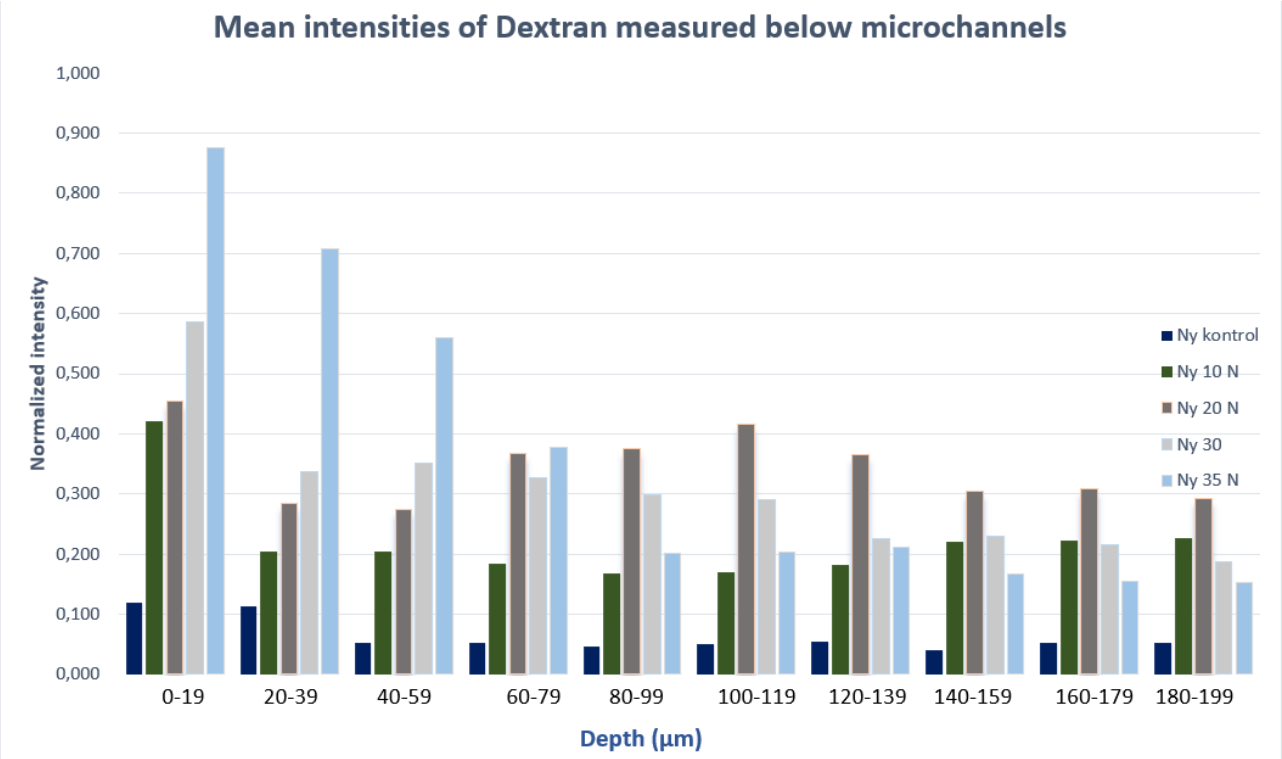


Figure 15: A bar chart presenting the normalized intensity of Dextran as a function of the depth of the skin in µm

Figure 15 displays how treating the skin with a dermaroller show a clear effect on penetration. Like figure 13, it is observed how a greater force increase the penetration of Dextran, as 35 N demonstrates to have the highest penetration until below 80 µm. Furthermore, it is observed that 20 N has the highest penetration out of all the measurements from 80 µm, which presumably is autofluorescence.

It is noticeable how the values for 30 N in figure 15 are lower than anticipated. To further investigate the penetration behavior of 30 N, a new graph was created with four random measurements presented (see Appendix II, Figure A3). It is seen how all four graphs have diverse behavior, with only one having a similar plot profile to the control in figure 15. This is an indication that the penetration of Dextran even varies among microchannels that are created by the same force. As mentioned in 4.2., images showed both dents and disrupted microchannels, and the penetration profile may vary, dependent on the microchannel. Likewise, to investigate the mean pattern of 35 N, four random measurements were plotted into on graph (see Appendix II, Figure A4). The graph also showed how the plot profiles vary, which may again be due to how well the microchannel has disrupted SC.

Likewise, the penetration of GFPM was investigated. Every mean set of force was plotted, with the number of particles as a function of the depth (See Appendix II, Figure A5). The graph illustrate how many particles of GFPM penetrate the skin and the graph supports results from figure 15, as 35 N demonstrates to have the most prominent penetration profile of GFPM (particles are observed below 140 µm). It is also observed that 10 N still has a rather unnoticeable penetration, which suggests that a force between 20 to 35 N or more is needed for substantial penetration enhancement of GFPM.

4.5. Analysis of dextran horizontally across the epidermis

Dextran was finally investigated for its ability to penetrate horizontally across the epidermis. Method illustrated on figure 11, section 3.6. show how a scale bar was used to investigate the intensity (arb.units) from the SC and 400 µm horizontally into the epidermis. 20 measurements for each set of forces was obtained. However only 14 measurements for 35 N were obtained due to damaged SC in the images (see Appendix III, Figure A6). The mean values for intensity were plotted in a bar chart as a function of the depth of epidermis (in µm) (see figure16).

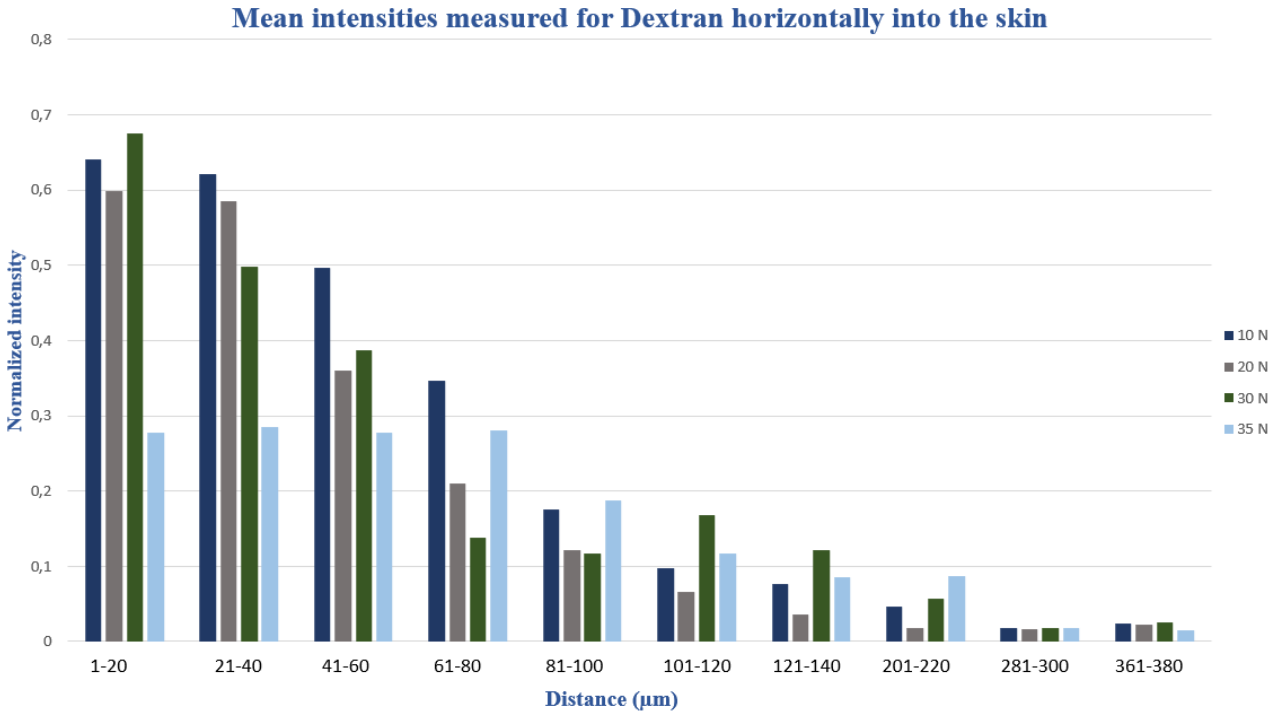


Figure 16:A bar chart illustrating the mean intensities measured for Dextran horizontally into the skin.

Figure 16 shows an overview of the results, by presenting selected values from 1-60µm, 91-100 µm, 131-140 µm, 171-180 µm and 191-200 µm and their respective normalized intensity (see Appendix IV, Figure A7 for every interval). The penetration of Dextran is noticeably higher from 1-40 µm compared to penetration after 131 µm. SC is 10-20 µm thick, so the results indicate that Dextran is still primarily found in the SC after 22 hours of allowed penetration [4]. The overall results shows that the penetration decreases as the value for distance increases. The results also show until below 91 µm, the mean values for 35 N are the greatest. Interestingly, both 10 N and 20 N has greater values than 30 and 35 N, however this may be a result of normalizing the data, and thereby not the true penetration profile for 10 and 20 N.

5. Discussion

5.1. The depth of the perforated holes

Nanoparticles have been investigated according to topical and transdermal drug administration. However, molecules' physiochemical properties determine whether they will be able to transport through the skin. Microneedling is one of several enhancement techniques, that are widely investigated according to optimization of both topical and transdermal administration of molecules.

However, microneedling is still under development and many factors are still to be researched. This project investigated the results of perforating human abdominal skin with a dermaroller at different forces, to see how deep microchannels the dermaroller would be able to generate and how it correlates to penetration of fluorescent drug analogues.

Table 3 presents the results of treating the human skin with forces 10, 20, 30 and 35 N. The results showed that 20 N had the lowest mean depth (calculated from 22 different samples) and 30 N had the highest mean depth (calculated from 21 different samples). Meanwhile, 10 N and 35 N had similar mean depths, with 35 N only being 2.08 µm deeper.

However, it was observed under investigation of the skin samples of 35 N, that some samples had a destroyed SC, which was detached from the rest of the skin on several of the images. This may be due to the large force applied, but it may also have occurred during preparation of the sample. As there is no literature found on which force may irreversible disrupt the SC, it is unknown what caused the destroyed cross sections.

The lack of measurable force samples with 35 N may have an influence on why the results from 35 N were similar to the results from 10 N. however, it is observed that a force of 10 N primarily made dents in the SC, while 35 N made properly perforated holes. This suggests, that the depth may not have a significant importance on the penetration, but it is more dependent on whether the SC is disrupted. Overall, results presented in Table 3 therefore indicate no direct correlation between the force applied and the depth of the observed microchannel.

Other studies performed on this given project are very limited, however *Kalluri et al*. (*2011*) investigated the microchannels created by a DermaRoller® on hairless rats. The study used LSCM and 0.2 µm sized FlouSpheres® to visualize the depth of the created microchannels, and results showed microchannels had an average depth of 152.5±9.6 µm (created by microneedles with a length of 770±8.9 µm). The results obtained from *Kalluri et al. (2011)* differ from the resulted shown in Table 3. The highest mean depth registered in Table 3 is 125.52±53.22 µm with applied force of 30 N. Even more noticeable is, how the needle length used in this thesis was an average of 1.5 mm, making it almost double the length compared to *Kalluri et al. (2011)* who used needles with a length of 770±8.9 µm.

Likewise has *Kunsche et al. (2009)* made an in vitro evaluation of skin perforation using DermaRollers® with sizes of respectively 150, 500 and 1500 µm. The samples were initially investigated using light microscopy which showed that no microchannels were visible using 150 µm microneedles. However, results displayed how using a Dermaroller® with a needle length of 1.5 µm would result in a microchannel with a size of 183.23 µm. This indicate that the length of the needle has a considerable influence on the depth of the microchannel.

Noticeable for both the studies of *Kalluri et al. (2011)* and *Kunsche et al. (2009)* is that they omit to mention which force was applied during the perforation process. It is therefore unknown what forces must be applied to achieve an average mean depth of 125.52 using a 770 µm needle or a mean depth of 183.23 µm using a 1.5 mm needle.

The results presented in Table 3 showed that there was no clear correlation between the applied force to the skin and the depth achieved. It is therefore arguable that the length of the needle is more relevant to achieve a deeper perforation, than the force applied. However, the depth may not be the only crucial factor when it comes to how fluorescent drug analogues penetrate the skin, as it may also depend on whether the SC has been disrupted or not. Therefore, further investigations should be performed to test the influence on how fluorescent drug analogues penetrate the skin.

5.2 Penetration of Dextran and Green Fluorescent Polystyrene Microspheres above the perforated holes

Conventional transdermal drug delivery is very limited to small drug molecules, due to their ability to diffuse through SC. However, promising investigations display that microneedling may be able to increase the skin permeability by up to 3 orders of magnitude [5]. The treatment is relatively painless, as it will not afflict any nerves, making this a painless route for administration in contrary to intravenous administration.

To examine how potential drug molecules may penetrate the skin, fluorescent labeled Dextran was used as a drug analogue and results were presented in figure 13. It is observed that the dermaroller increases the penetration of Dextran. The penetration is showed to be the most abundant for 35 N until below 100 µm, while 10 N was observed to have an insignificant penetration activity. Furthermore, 20 N showed to have similar penetration profile as 35 N, but the intensities measured until below 80 µm was lower than the penetration measured for 35 N. This indicate that a force between 20-35 N must be applied for successful transdermal drug delivery. Although the dataset for 30 N shows to have a low penetration until below 100-139 µm it, it also the one having the highest penetrations from 180 to 399 µm, indicating that the penetration of Dextran can occur into deep parts of the skin sample. These results suggest great promises for enhanced transdermal delivery, but they also suggest, that different forces can be selected to deliver drugs to different depths, which can also be useful knowledge for optimal drug administration.

As for GFPM, the results also showed promising results for respectively 30 and 35 N. Figure 14 shows how there is still an apparent number of particles present at 150 µm, suggesting that perforating the skin, has greatly improved the penetration abilities for the particles. This may indicate that microneedling is beneficial for the permeation of nanoparticles in general.

Zhang et al. 2010 investigated how poly(D,L-lacic-co-glycolic-acid) (PLGA) nanoparticles behaved in human skin treated with microneedles and a applied force around 15 N. The skin samples were investigated using LSCM to understand how the PLGA nanoparticles behaved through the microchannels. Results suggested that the percentage of the applied dose increased, not only because of the the microchannels, but also according to time interval, the samples were left to penetrate. Therefore, the highest percentage of applied dose in the dermis was achieved within a 48 hour period.

Zhang et al. 2010 also investigated the importance of the size of nanoparticles. Results also showed that small nanoparticles had a greater chance to penetrate the skin compared to larger nanoparticles.

The results presented by *Zhang et al. 2010* is to a certain extent in agreement to results presented in figure 13 and 14. *Zhang et al. 2010* observed the flourescent particles down to at a depth below 68.32 µm using a force of 15 N, while this experiment showed to have penetration of Dextran for 20, 30 and 35 N below 100 µm and GFPM also showed to have a significant number of particles present below 100 µm in forces of 20, 30 and 35 N samples. The results of this experiment, along with the results obtained på *Zhang et al.* show, that the penetration enhancement method, microneedling, has an ability to improve the diffusion of nanoparticles across SC and towards dermis. The human abdominal epidermis is approximately 60 µm thick, suggesting that both Dextran and GFPM can diffuse into the dermis after microneedling with forces 20, 30 and 35 N [9].

5.3. Penetration of dextran and GFPM beads measured under the perforated holes

Figure 15 illustrates how Dextran behaves from the end of perforated microchannel and into the dermis. This investigation gave a better insight of how fluorescent labeled Dextran and GFPM penetrate the skin. The results showed promising similarities to measurements obtained above the microchannel. The data sets were normalized to take into consideration the intensities for each dataset may differentiate, which will give ambiguous results. The normalized data showed, how 35 N still has the highest measured penetration compared to all the other forces, until below 60 µm. The penetration with a force of 30 N is observed to be unchangeable in the interval 120-199 µm, however, this may occur because the data was normalized and not because the penetration is constant. 20 N showed to have an increasing penetration below 120 µm, which indicate autofluorescence in this interval. Lastly, 10 N showed to have an inconsiderable penetration, which was also observed on figure 14. This is again an indication, that forces 20-35 N could be used for transdermal drug delivery.

5.4. Investigation of Dextran's ability to penetrate horizontally across epidermis

Lastly, figure 16 presents how Dextran penetrate horizontally from the perforated hole and into epidermis. There is no control for reference, as the presence of microchannels are needed for measurements. The results were normalized as the intensity at Dextran may vary from every set of force. Figure 16 showed, that unlike the previous discussed results, 35 N does not have the largest penetration, making the results differentiate from the previous discussed results. However, this may be due to normalization of the data and does not indicate, that penetration seen from 35 N measurements were unnoticeable, as there is measurable amount of penetrated Dextran present at 201 µm. The results overall showed, that not only will Dextran penetrate downwards towards dermis, but Dextran also penetrates horizontally along the epidermis. This is valuable knowledge regarding treating diseases that occurs in the skin layers, such as epidermis. This gives an improved insight of how the force applied may have an influence of how fluorescent drug analogues penetrate the skin both downwards but also horizontally. Both Figure 13 and 15 suggest, that a force with 35 N gives the largest penetration below SC and horizontally into the skin sample. 35 N may therefore be the optimal force, if the aim is to reach systemic circulation. However, if the treatment is used for more local skin diseases, then forces 20 and 30 N may optimal, as the fluorescent drug analogues had a lesser overall penetration shown in figure 13 and 15, but they showed similar results to 35 N, when measuring the intensity horizontally. Nevertheless, results showed that perforating skin by micorneedling would improve the penetration of Dextran downwards into the dermis but also horizontally further into epidermis.

6. Conclusions

This project focused on investigating the penetration the fluorescent particles 70000 MW Dextran and 1.0 µm Green Fluorescent Polymer Microspheres on human skin perforated with microneedles (using a dermaroller device) at different forces. The cross sections of skin samples obtained were investigated using Laser Scanning Confocal Microscopy for respectively the depth of every microchannel and the penetration of the fluorescent particles.

The size and depth of the microchannels in the skin were investigated and results showed no clear correlation between the depth of a microchannel and the force applied to the skin sample. However, 10 N showed to be more likely to make dents rather than to disrupt the stratum corneum.

The intensities of fluorescence in the skin samples were analyzed by imaging the skin samples. Analysis of Dextran both above and below the microchannels showed how applying a force of 35 N would result in the highest overall penetration close to stratum corneum, but also further into the skin sample. However, forces 20, 30 and 35 N displayed that Dextran could penetrate all the way into the deeper part of dermis. Green Fluorescent Polymer Microspheres also showed a similar pattern to Dextran, as 30 N and 35 N displayed the highest number of particles below 150 µm into the skin. It was also discovered, that Dextran does not only penetrate downwards to the deeper parts of the skin, but also horizontally into the epidermis. Lastly, 10 N was found to not be a large enough force to make a microchannels that could optimize penetration for or Dextran or Green Fluorescent Polymer Microspheres.

As for the intact skin, the fluorescens did not penetrate the skin barrier, however some Dextran was observed. This confirms that treating the skin with microneedling enhances the penetration of both Dextran and Green Fluorescent Polymer Microspheres.

In conclusion, microneedling shows to be a promising enhancement method for transdermal penetration. However, many parameters effect the size of the microchannels and the penetration of fluorescens, and not only the force applied, but also the needle length and which fluorescens is applied. Thus, further in vitro/in vivo experiments should be conducted, investigating the significance of both the force applied, but also the needle length of the Dermaroller.

Acknowledgement

First and foremost, I would like to thank my supervisor, Jonathan Brewer, for giving me this opportunity and welcoming me into your group. Your guidance and supervision throughout this whole project was more than I could ever have asked for.

Secondly, I would like to thank Vita Solovyeva for always helping me, no matter what time of the day it may be.

To Bjarne Thorsted: Thank you for always answering my questions and for your cheerful mood. Nobody has a gloomy day when you are around.

Furthermore, I would like to thank Helle Gam-Hadberg and Brian Bjarke Jensen for offering great advice and helping me throughout this whole project.

Lastly, I would like to thank Ditte Bork Iversen. These last four months has truly been a pleasure due to your great company and companionship.

References

[1] Banga, A.K. (2011) Transdermal and Intradermal Delivery of Therapeutic Agents: Application of Physical Technologies, CRC Press, page 1-18.

[2] Jablonski, N. G (2006), Skin: A Natural History, Univeristy of California Press.

[3] Aulton, M.E and Taylor, K (2013), Aulton's Pharmaceutics: The Design and Manufacture of Medicines, Churchill Livingstone/Elsevier, 676-688

[4] Marieb, Elaine N. Essentials of Human Anatomy and Physiology, San Francisco, CA: Perason/Benjamin Cummings, 2009, 140-147

[5] Escobar-Chavez, J.J., Bonilla-Martinez, D., Villegas-Gonzalez, M.A., Molina-Trinidad, E., Casas-Alancaster, N. and Revilla-Vazquez, A.L. (2011) Microneedles: a valuable physical enhancer to increase transdermal drug delivery. J Clin Pharmacol 51(7), 964-977.

[6] Szunerits, S. and Boukherroub, R. (2018) Heat: A Highly Efficient Skin Enhancer for Transdermal Drug Delivery. Front Bioeng Biotechnol 6, 15

[7] Haq, M.I., Smith, E., John, D.N., Kalavala, M., Edwards, C., Anstey, A., Morrissey, A. and Birchall, J.C. (2009) Clinical administration of microneedles: skin puncture, pain and sensation. Biomed Microdevices 11(1), 35-47.

[8] Coulman, S.A., Anstey, A., Gateley, C., Morrissey, A., McLoughlin, P., Allender, C. and Birchall, J.C. (2009) Microneedle mediated delivery of nanoparticles into human skin. Int J Pharm 366(1-2), 190-200.

[9] Zhang, W., Gao, J., Zhu, Q., Zhang, M., Ding, X., Wang, X., Hou, X., Fan, W., Ding, B., Wu, X., Wang, X. and Gao, S. (2010) Penetration and distribution of PLGA nanoparticles in the human skin treated with microneedles. International Journal of Pharmaceutics 402(1), 205-212.

[10] Badran, M.M., Kuntsche, J. and Fahr, A. (2009) Skin penetration enhancement by a microneedle device (Dermaroller) in vitro: dependency on needle size and applied formulation. Eur J Pharm Sci 36(4-5), 511-523.

[11] Dermaroller HC902, accessed 21 may 2018, https://www.original-dermaroller.de/en/home-skin-care/products/7/dermaroller-hc902

[12] Iriarte, C., Awosika, O., Rengifo-Pardo, M. and Ehrlich, A. (2017) Review of applications of microneedling in dermatology. Clin Cosmet Investig Dermatol 10, 289-298.

[113] Dragicevic, N. and Maibach, H.I. (2016) Percutaneous Penetration Enhancers Chemical Methods in Penetration Enhancement: Nanocarriers, Springer Berlin Heidelberg, page 304

[14] Kalluri, H., Kolli, C.S. and Banga, A.K. (2011) Characterization of Microchannels Created by Metal Microneedles: Formation and Closure. The AAPS Journal 13(3), 473-481.

[15] Kaszuba, M., Corbett, J., Watson, F.M. and Jones, A. (2010) High-concentration zeta potential measurements using light-scattering techniques. Philos Trans A Math Phys Eng Sci 368(1927), 4439-4451.

[16] What is Zeta Potential?, Brookhaven Instruments Corporation, accessed 21 may 2018, https://www.brookhaveninstruments.com/what-is-zeta-potential

[17] Parts of a Franz Cell, PermeGear, accessed 21 may 2018, http://permegear.com/parts-of-a-franz-cell/

[18] Claxton, N.S., T.J. Fellers, and M.W. Davidson. Laser scanning confocal microscopy, accesses 22. May 2018, http://zeiss-campus.magnet.fsu.edu/referencelibrary/pdfs/ZeissConfocalPrinciples.pdf

[19] Wilhelm, S., et al. Confocal Laser Scanning Microscopu: Principles, accessed 22. May 2018, http://zeiss-campus.magnet.fsu.edu/referencelibrary/pdfs/ZeissConfocalPrinciples.pdf

[20] Dextran, Texas Red™, 70,000 MW, neutral, accesses 22 May 2018, https://www.thermofisher.com/order/catalog/product/D1830?SID=srch-srp-D1830

[21] Flouspeheres™ Polystyrene Microspheres, 1.0 µm, yeollow-green fluoresvent (505/515), for tracer studies, accesses 22 May 2018, https://www.thermofisher.com/order/catalog/product/D1830?SID=srch-srp-D1830

[22] OECD, Guidance Document for the Conduct of Skin Absorption Studies, 2004: OECD Puclishing, Paris, https://www.oecd-ilibrary.org/docserver/9789264078796-en.pdf?expires=1526949112&id=id&accname=guest&checksum=5AFAF2290BB539D8B032AE5FFDA47D08

[23] El-Domyati, M., Abdel-Wahab, H. and Hossam, A. (2018) Microneedling combined with platelet-rich plasma or trichloroacetic acid peeling for management of acne scarring: A split-face clinical and histologic comparison. J Cosmet Dermatol 17(1), 73-83

[24] Shah, P., Desai, P., Patel, A. and Singh, M. (2012) Skin permeating nanogel for the cutaneous co-delivery of two anti-inflammatory drugs. Biomaterials 33(5), 1607-1617.