TRANSDERMAL PENETRATION OF DRUG-ANALOGUES BY THE USE OF A DERMAROLLER

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Content

Abstract

The aim of this study, was to investigate the ability to puncture the stratum corneum layer and increase the penetration of drug-analogues as a consequence of applying different forces to the dermaroller. The hypothesis was that an optimal drug skin penetration would occur at a specific force range. The drug-analogues used in this project were beads (1.0 μm) and dextran (0.02 μm), both molecules cannot passively cross the skin barrier, because both of them have a molecular weight larger than 500 Da. The dermaroller contains small needles that disrupt the stratum corneum barrier, this is an advantage because transport across the stratum corneum compose a big challenge when delivering drugs transdermally.

In this in vitro experiment skin samples obtained from plastic surgent were used. Skin samples were pre-treated with a dermaroller applied to the skin with forces of 10, 20, 30 and 35 N. The fluorescence labelled drug-analogues were added to the skin surface and the penetration depth was measured. Detection of the drug-analogues was done by using a confocal laser scanning microscope in both horizontal and vertical directions.

Horizontal penetration was only measured for dextran. The longest horizontal penetration length was seen when skin samples were treated with a force of 35 N, which gave a penetration length of 131-140 μm. The maximum vertical penetration depth of dextran was found to be 251-271 μm, when skin samples were pre-treated with 30 N. For beads, the maximum vertical penetration depth was measured to be 280 μm when skin samples were treated with 30 N. The measurements show that the drug-analogues could be successfully delivered below the stratum corneum to the epidermis and dermis. All the measurements were started at the surface of the stratum corneum and down to the epidermis and dermis layer.

Taken together our results showed that to insure penetration of the drug-analogues tested, the force applied to the dermaroller needed to be between 30-35 N. When this force was applied the largest penetration of test molecules and the highest frequency of penetrated holes (punctured skin) were seen. This indicates that the force applied to the dermaroller is important and working further on this method could pave the way to enhance drug penetration into the skin in the future.

Dansk resume

Formålet med dette studie var at undersøge, om en dermaroller, anvendt på huden med forskellige tryk målt i newton, ville resultere i forskellig evne til at bryde stratum corneum og derved resultere i forskellig penetration af forskellige lægemiddel-analoger. Hypotesen for studiet var, at ved et specifikt tryk ville man opnå en optimal penetration over huden.

De lægemiddel-analoger, der blev benyttet i dette projekt var beads (1,0 µm) og dextran (0,02 µm). Der gælder for begge molekyler, at de ikke kan penetrere hudbarrieren ved passiv diffusion, da de begge har en molekylevægt større end 500 Da. Funktionen af en dermaroller er at punktere stratum corneum, som netop udgør den vigtigste barriere, når man ser på transdermal lægemiddel transport.

I dette in vitro forsøg anvendtes overskudshud indhentet hos plastikkirurger. Hudprøverne blev først behandlet med en dermaroller, hvorpå der blev pålagt et tryk på henholdsvis 10, 20, 30 og 35 N. Fluorescens mærkede lægemiddel-analoger blev herefter påført hudoverfladen, og penetrationslængden gennem huden blev målt. Påvisning af lægemiddel-analogerne i huden skete ved brug af et konfokal laser scannings mikroskop. Penetrationen blev undersøgt i både den horisontale og den vertikale retning. Horisontal penetration blev kun undersøgt for dextran. Den længste horisontale penetrationslængde for dextran blev målt efter hudprøverne var blevet udsat for et tryk på 35 N fra dermarolleren. Den længste penetrationslængde lå i intervallet 131-140 µm. Den maksimale vertikale penetrationslængde for dextran blev målt til at ligge i intervallet 251-271 µm ved et tryk på 30 N. For beads blev den maksimale penetrationslængde målt ved 30 N med en dybde på 280 µm. Målingerne viste altså, at det var muligt at få lægemiddel-analogerne ind i huden og dermed over stratum corneum og ned til den dybe del af epidermis og dermis. Alle målinger blev foretaget fra hudoverfladen og ned gennem epidermis og dermis.

Den overordnede konklusion på dette projekt var, at det tryk, der skulle påføres dermarolleren for at være sikker på at opnå penetration af lægemiddel-analogerne, var 30-35 N. Dette gjaldt både når man ønskede at opnå den længste penetration af lægemiddel-analogerne og den højeste frekvens, hvormed, der blev lavet huller i huden. Dette indikerer, altså at trykket på dermarolleren er af betydning, og i fremtiden vil det være væsentligt yderligere at undersøge denne teknik, for derigennem at udvikle en ny metode til at øge transporten ind i huden af topikalt administrerede lægemidler.

Abbreviation list

PBS buffer = Phosphate buffered saline

LSM = Laser scanning microscopy

SD = standard deviation

SEM = Standard error of the mean

1. Introduction

Optimization of transdermal drug delivery has in recent years become a growing research field. With an average skin area of 1,8 m^2 in an adult person, it forms the largest organ of the body and hereby also a major target for drug delivery (1). Among the different methods of optimizing transdermal drug delivery, improvement of drug penetration with microneedles has recently gained much attention (2).

Transdermal drug delivery functions as a good alternative to oral administration. It harbours many advantages such as avoiding first passage metabolism in the liver, less systemic exposure and thereby often less side effects and delivery of the drug to the target organ when used for treating skin disease. It also serves as an alternative for subcutaneous injections. Subcutaneous injections can lead to a problem especially for the developing countries, where the risk of disease transmission is of great interest because of needle-reuse. Furthermore injections are also painful for the patient, leading to low patient compliance (3).
Enhanced topical drug penetration by the use of a dermaroller with microneedles overcomes some of these problems. When using the transdermal route, the dose can be self-administered which may lead to enhanced patient compliance but also because of the microneedles ability to function almost painless. There is also a great interest in using microneedles to deliver macromolecules, peptides and vaccines, such as insulin and influenza vaccine. The advantage of microneedles is its capability of making holes in the stratum corneum layer of the skin barrier. This leads to the penetration of a variety of drugs and the ability to reach deeper part of the skin and even the systemic circulation (4, 5).

The optimal force to be applied when using the dermaroller have still not been investigated. It is expected to be important when a successful uniform drug delivery is achieved. It is therefore of great interest to further study what force on the dermaroller leads to the most optimal drug delivery. This question can be further studied by using confocal laser scanning microscopy (LSM) to measure the penetration distance of drug-analogues, and the hole depth in the stratum corneum (6). In this project the drug-analogues used to penetrate the skin are dextran (0.02 μm) and beads (1.0 μm), both are not able to passively penetrate the stratum corneum barrier, due to a weight higher than 500 Da (7).

The aim of this study, was to investigate the ability to puncture the stratum corneum layer and increase the penetration of drug-analogues, as a consequence of applying different forces to the dermaroller.

The hypothesis of this study was that within a specific force range, multiple holes in the stratum corneum will appear leading to an optimal skin penetration.

The perspective of this study is that by identifying a critical force, it will in the future be possible to standardize the use of a dermaroller with respect to the force applied, and hereby optimize the transdermal drug delivery route.

2. Theory

2.1 The skin barrier and criteria for transdermal drug delivery

The skin consists of three layers; called epidermis, dermis and subcutis which all together provides a barrier for the human body. Some of the main functions of the skin is; to regulate fluid and electrolyte balance, regulate body temperature, serve as an immunological and metabolic organ and to function as a barrier against heat, cold, trauma, radiation, infections and toxicological substances (8).

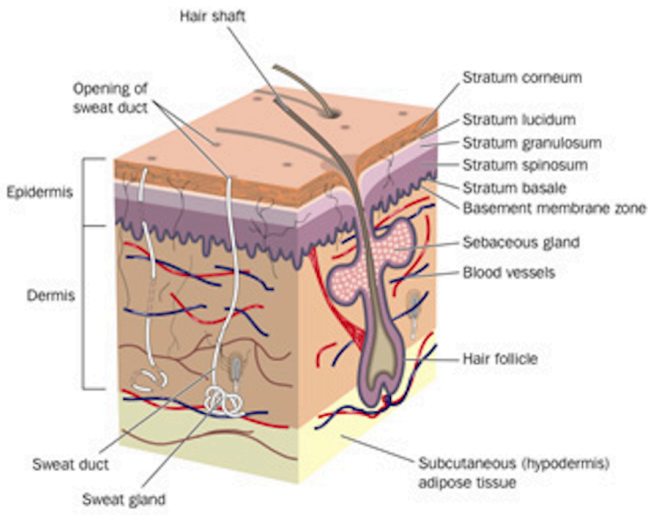


Figure 1 The overall structure of the skin, consisting of epidermis, dermis and subcutis (9).

The epidermis is approximately 0,075 mm thick depending on where on the body it is measured. As seen in Figure 1, epidermis is the outermost layer and consist of stratum corneum, stratum granulosum, stratum spinosum and stratum basale (8). When it comes to drug delivery through the

skin, stratum corneum is the most important layer. It consists of corneocytes packed in lipid bilayer, which leads to a very lipophilic barrier (10). The thickness of the stratum corneum layer varies depending on where it is measured, in general it consists of 18 to 21 cell layers, which gives an overall thickness of 10-30 μm in most skin regions (11, 12).
When using microneedles, it is the stratum corneum barrier which is penetrated (10).
The dermis is the layer just underneath the epidermis, it is 3-5 mm thick and contains mainly of collagen and elastin. In the dermis the capillaries, arterioles and venules are located, and through here the drug can reach the systemic circulation. This leads to a constantly low concentration in the skin, and therefore a concentration gradient that makes it possible for the drug to penetrate from the skin surface to the bloodstream and thereby the entire body.
The subcutaneous tissue is believed to have a less important function considering transdermal drug delivery. It mainly consists of adipose cells, fibroblasts, nerve endings and macrophages (1).

When considering transdermal drug delivery, it is not enough only focussing on the physical barrier of the skin, the physicochemical properties of the drug is also of great interest, when an optimal therapeutic effect is sought.
For a drug to passively cross the skin barrier four guidelines have been established. These guidelines have been developed empirically, by studying which drugs are on the market.
The four main guidelines are;

- The molecular weight cannot exceed 500 Da
- The partition coefficient, log P, must be between 1-4
- The molecule should have a low melting point
- The daily dose given should not exceed more than 10 mg/day (13).

If these criteria are fulfilled the drug will have greater ability to passively penetrate the skin barrier. However, applying to all the rules provides many limitations in developing new pharmaceutical drugs, and the search for alternative transdermal drug delivery routes is necessary. Previous studies have shown that a dermaroller can overcome the rule of a molecular weight of maximum 500 Da and instead manage to deliver example protein and vaccines with a much greater molecular weight than 500 Da. When making microchannels, dermarollers have also shown the ability to make an aqueous transport pathway, which makes it possible for hydrophilic molecules to by-pass the stratum corneum (7).

2.2 Dermaroller

A dermaroller is a small device with a varying number of needles, and with different needle sizes. The needles are attached to a cylinder which is able to roll over the skin making small microchannels.

Figure 2 A dermaroller with 540 microneedles.

The main function of the dermaroller is to disrupt the stratum corneum barrier, and thereby enhance the penetration of drugs through the epidermis layer, for delivering of drug locally in the skin or to the systemic circulation. Thus, it is important to avoid the needles to traumatize or even get into contact with blood vessels and nerve fibers (7, 14). This is done by making small microchannels in the skin (15). The dermaroller is therefore a very popular device. It is painless, easy to use, and the production cost is cheap. Furthermore, a dermaroller is already used against acne scar, acne, skin rejuvenation and stretch marks, where it has shown great results (16, 17).

Many studies using microneedles for enhancing transdermal drug delivery have already been conducted. The dermaroller improves the penetration of a verity of drugs including a range of low molecular weight drugs, proteins, drug-loaded nanoparticles and vaccines, all drugs that normally do not penetrate the stratum corneum layer (3, 18).

Studies have also shown that microchannels caused by microneedles can increase skin permeability for up to one day. After this the microchannels in the skin closes and the skin end up being intact (15).

2.3 Franz diffusion cell

When measuring penetration of a drug-analog, a Franz diffusion cell system is one possible choice. A Franz diffusion cell consists of a donor chamber, membrane, heater/ circulator, sampling port and a receptor chamber, as seen on Figure 3. At the donor chamber, the formulation to be tested is added. A membrane is separating the donor chamber from the receptor chamber. Different membranes can be used including human skin. To obtain conditions corresponding to the human

physiology, a phosphate buffered saline, with a pH of 7.4, is often used in the receptor chamber and a temperature at 32°C is maintained during the experiment (19). After a defined time, the membrane could be removed for further investigation.

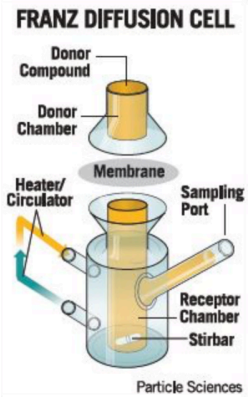


Figure 3 Franz diffusion cell (19).

2.4 Confocal laser scanning microscopy

The confocal LSM is widely used to detect and localize fluorescence's (20). Some of the advantages with confocal LSM is the ability to analyse a sample with a thickness of up to 100 µm. This is done by analysing the sample in different planes or levels, with a movement along the axial direction, named the optical axis (z). This is called optical sectioning and is obtained by scanning the sample point by point or plane by plane. When this has been done the whole image is reconstructed giving rise to a 3D data set.
Likewise, it is also possible with confocal LSM to analyse samples with two or more fluorescence's because of the ability to separate the different signals. This prevent the multiple fluorescence's to mix and thereby avoid an image with mixing colours (20, 21). However, one of the absolute greatest advantages is the ability to eliminate the out-of-focus-light (22).

Confocal LMS as well as normal fluorescence microscopy is based on excitation and emission of fluorescence which can be explained by Jablonski-diagram.

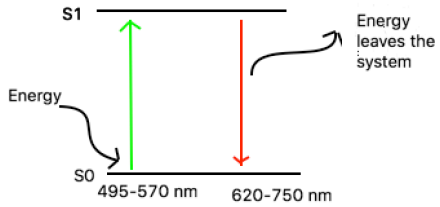


Figure 4 A diagram of green light which by excitation enter the S_1 state. And later by emission decay and emit a photon which makes the light appear as red, and the electron return to the S_0 state.

Figure 4 illustrates that energy of the electromagnetic radiation, forces an electron to be absorbed and thereby enter the S_1 state from the ground state, S_0. An energy transfer has occurred.
After 10^{-9}-10^{-8} seconds the electron decay and emit a photon. The photon will have lower energy, leading to a radiation with a longer wavelength, allowing it to be separated from the excitation light. The wavelength corresponds to a particular colour and is the one observed in the microscope (23).

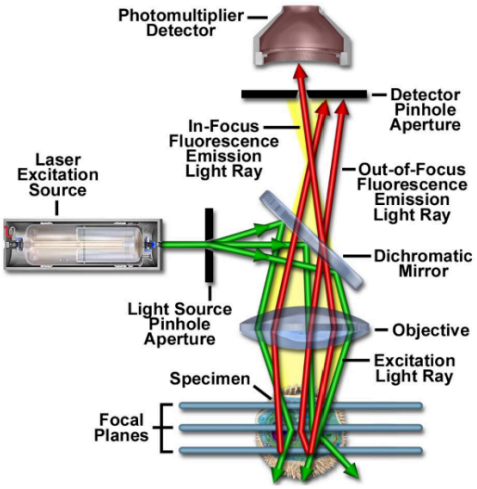


Figure 5 Diagram of confocal LSM. Excitation of green light hits the objective and is directed to the specimen. Later only the light in focus will pass through the pinhole and reach the detector (21).

Figure 5 shows the confocal LSM. A laser excitation source travels through a pinhole and hits a dichromatic mirror, which reflects the light and send it to the objective. The objective focuses the light onto the specimen in a decided focal plan. Afterwards the emitted light hits an emission filter, which prevent the excitation laser light to pass and only allows the emitted light to pass. The emission beam from the specimen passes then through the pinhole on the detector, which only lets the light from the focus plane pass and reach the detector. The light coming from above or below the focal plan on the specimen, will be rejected by the pinhole aperture, and will not reach the detector. When the microscope focus is moved, light from the new focal plane can pass through the pinhole. This enables collection of clear focused optical sections at different depths in the sample.

The pinhole aperture is one of the very important things that differs confocal microscopy from fluorescence microscopy (20, 21).

3. Materials and methods

3.1 Materials used to detect penetration length

FlouSpheres® Carboxylate-modified microspheres, dextran, of 0.02 µm and polymer microspheres green fluorescing, beads, of 1.0 µm were diluted with phosphate buffered saline (PBS) with a pH of 7.4, to a concentration of 0.1 mg/ml and 1 mg/ml, respectively. Both fluorophores were purchased from Thermo Fisher Scientific.

Table 1 Compounds labelled with fluorophores used to detect the penetration length in the skin. The values for labelling, excitation/emission and molecular weight was collected from the webpage of the producer (24, 25).

Compound	**Label**	**Excitation/emission, nm**	**Molecular weigh**	**Molecular size, µm**
FlouSpheres® Carboxylate-modified microspheres, Dextran	Texas Red	595/615	70,000 Da	0.02
Polymer microspheres green fluorescing, Beads	Blue-green fluorescent	430/465		1.0

The dermarollers used were *Derma Roller system* with 540 titanium needles. Each needle had a length of 1.5 mm. The dermaroller was formed as a cylinder with a total area of 9.3 cm^2, 9 rows of microneedles and a distance in every row between the microneedles of 0.5 mm.

3.2 Preparation of skin samples

Skin obtained from patient undergoing plastic surgery, because of obesity at Odense University Hospital, was used for further investigations. The laboratory work was repeated twice with skin

from to different donors. The two batches of skins were obtained from two women aged 48 and one with unknown age, respectively. Both skin samples were collected from the abdomen. From the fresh skin the subcutaneous fat tissue was removed with a scalpel, and afterwards stored in the freezer at -80 °C for a couple of days before use. Five square skin samples with a size of approximately 4x4 cm were cut for subsequent treatment with the dermaroller.

3.3 Skin penetration with dermaroller

Five different skin samples were prepared. One sample served as a control with no use of dermaroller and the other four were treated with the dermaroller with a pressure of 1, 2, 3 and 3,5 kg, respectively. The weight applied could be converted to force measured in newton (N) by Newtons law, which states that $F = m * g$. F is the force applied in newton, with the unit $kg * m * s^{-2}$, m is the mass in kg applied to the skin and g is the gravitational force at 9.83 N/kg. Conversion from kg to newton can be seen in Table 2.

Table 2 Conversion of kg to newton. The values correspond to the values used in this particular project.

Kg	**Newton, *N***
0	0
1	10
2	20
3	30
3.5	35

The skin samples were placed on a weight, stretched out with needles on a flamingo plate and the dermaroller were rolled over the skin with the selected pressure. The dermaroller rolled over the skin three times, always in the same direction.

After the force applied, the five samples were moved to the Franz diffusion cells with the stratum corneum facing the donor chamber. Approximately 15 ml of PBS buffer was added to the Franz diffusion cells receptor chamber. The samples were tightened and a magnet in the PBS buffer, adjusted to 500 rpm, were added to make sure stirring took place.

3.4 Adding the fluorophores, freezing and cutting the skin samples

200 µL of dextran together with 100 µL of beads were added to the surface of the skin sample in the donor chamber in the Franz diffusion cell. The two solutions were mixed with a volumetric pipette, and the whole setup was covered with tin foil to avoid evaporation. Each Franz diffusion cell had an area of 3.14 cm^2.

After 22 hours, the skin samples were removed from the Franz diffusion cells. Samples were cut into four smaller pieces, placed in a tin foil boat and submerged in a porcelain beaker with 2-methylbutane surrounded with liquid nitrogen. Afterwards the samples were stored in a -80 °C freezer before cutting. The samples were cut using a cryotome Thermo Scientific with a temperature of -25 °C. Every skin preparation was cut with a 25 µm width.

3.5 Confocal microscopy

The skin samples were analysed using a Leica SP8 CARS microscope. The objective used for analysing the samples were HC PL APO CS2 10x/0,40 DRY, with the HyD detector and the white light laser turned on. The dextran was measured at a wavelength of 590 nm with a laser intensity of 36 %.

The beads were measured at a wavelength of 485 nm with a laser intensity of 11.4 %.

Each picture was collected with a format of 1024x1024, a line accumulation of eight, a line average of one and a zoom at 1.25 so comparison of all images were possible. The images used for illustration in this bachelor project were obtained with a format of 3776x3776.

3.6 Data processing

The data processing was carried out in the program ImageJ.

3.6.1 Measuring the impression or hole depth in the skin

When using the dermaroller sometimes only an impression with no puncturing of the stratum corneum was seen instead of a hole. When measuring the depth of the impression/hole from the dermaroller, no distinction was made for these two, when looking at the results.

For every batch the impression or the hole depth was measured from the surface of stratum corneum to the bottom of the impression/hole. The measurements were always started at the lowest side of the stratum corneum as seen in Figure 6. Figure 6 is an image of the stratum corneum layer

where a force of 20 N was applied by the dermaroller and only an impression of the skin was seen. The red colour corresponds to dextran.

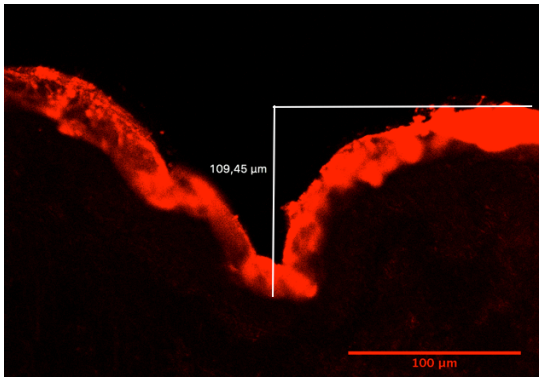


Figure 6 Image of skin obtained from abdomen where the force applied with the dermaroller correspond to a pressure of 20 N. The depth of the impression is measured from the surface of the lowest stratum corneum to the lowest part of the impression. After using the dermaroller the skin sample had been treated with dextran.

3.6.2 Measuring the penetration length of dextran and beads

When measuring the intensity of dextran and the number of particles of beads a rectangle box was drawn with a height of 2 µm. The measurement was started at the top of the lowest part of the stratum corneum surface, as seen in Figure 6, and continued throughout the skin for 200 steps, giving a depth of 400 µm. Although for dextran only data until a depth of 355 µm where able to be detected by the program ImageJ. Measurements with the rectangle box can be seen in Figure 7.

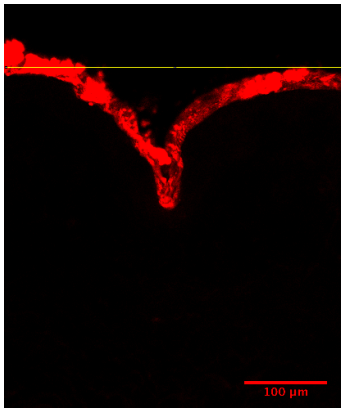


Figure 7 Measuring the intensity of a skin sample from the top of the stratum corneum treated with a force of 20 N and dextran. The box measuring the image has a height of 2 µm and measure 200 steps, which correspond to 400 µm.

Likewise, measurements of the penetration depth from the end of the hole was also carried out. The rectangle box was instead placed at the end of the hole and continued throughout the skin for 100 steps corresponding to a depth of 200 µm.

At last measuring the horizontal penetration was also carried out, where a measuring bar was placed with a distance of 200 µm from the inside of the impression/ hole of the stratum corneum as seen in Figure 8. The measurement was started halfway through the hole.

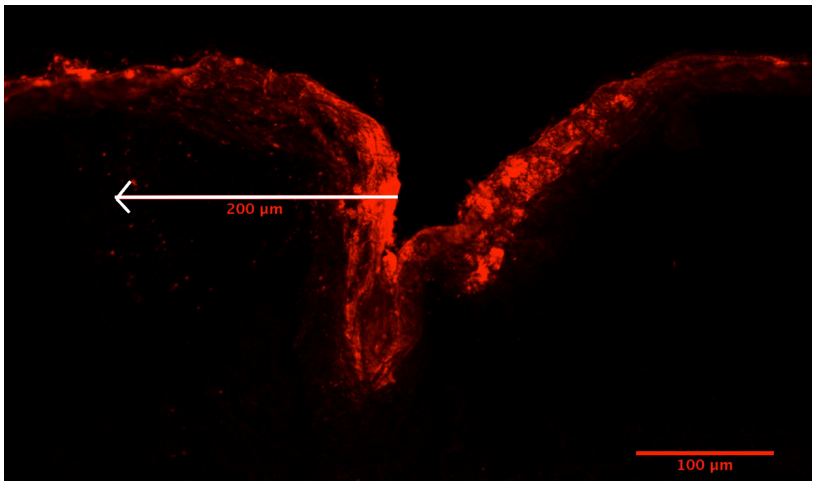


Figure 8 Measuring the horizontal intensity of a skin sample treated with 30 N and applied with dextran. The horizontal penetration is measured at a distance of 200 µm from the inside the hole, shown by the white arrow.

4. Results

4.1 Impression or hole depth as a result of the force applied to the skin with the dermaroller

When using the dermaroller, it was applied to the skin with different prespecified forces. The mean value of the force and standard deviation (SD) can be seen in Appendix 1, Table 4.

When the dermaroller was applied with either 10, 20, 30 or 35 N, 20, 23, 21 and 14 holes or impressions respectively, were studied further. The number of impressions or holes with different depths measured from the skin surface were counted and the results are shown in four histograms in Appendix 2, Figure 18. It is clearly seen that increasing the force by which the dermaroller was applied to the skin did not lead to increased impression/hole depth.

The mean hole depth ±SD as a result of the force applied with the dermaroller is also measured for the skin samples treated with the force of 10, 20, 30 and 35 N (Figure 9). A great variation of the hole depth is seen and no clear correlation or difference between the hole depth and the different forces applied to the dermaroller is observed.

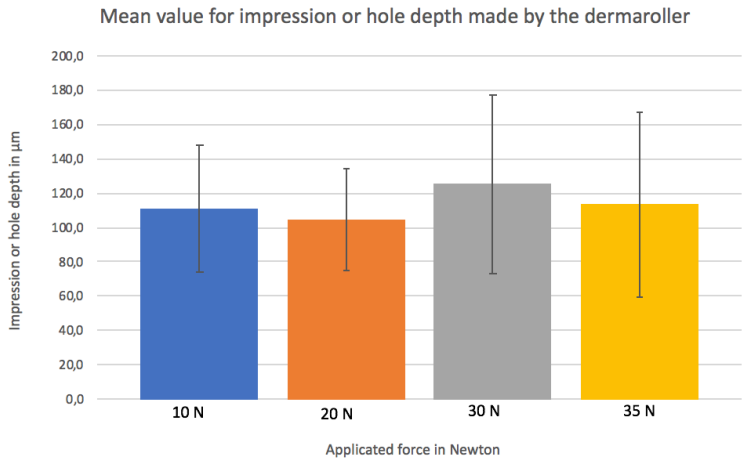


Figure 9 Shows the mean±SD of the impression or hole depth of the skin as a result of the different forces applied to the dermaroller. Forces of 10, 20, 30 and 35 N have been applied to the skin surface of four different samples. n is the number of holes or impressions studied (n_{10N}= 20, n_{20N}=23, n_{30N}=21, n_{35N}=14).

A pressure of 20 N appears to induce the smallest impression/hole depth (104.8±29.9 µm) whereas 30 N lead to the greatest depth (125.5±52.0 µm), but the difference is very low and most likely not relevant. With the calculated SD value, no clear observed differences between the depth of the impression/hole compared to the forces applied is seen, although a typical depth of 115 µm is observed.

Only 14 measurements were obtained for the samples tested with 35 N, which is lower than the number of measurements obtained with 10, 20 and 30 N (see Figure 9). This is because at the first skin batch, the stratum corneum layer was damaged and ripped off and therefore the results were excluded. This also affects the number of measurement with 35 N in the subsequent experiments. An image of the damaged skin barrier can be seen in Appendix 3, Figure 19.

After treatment of the two batches, the needles were checked in a stereo microscope, showing needles with varying sharpness. Some were bend down while others were still intact. Figure 10 is an image of the differences in the needle sharpness after the dermaroller had been used three times on ten different skin samples.

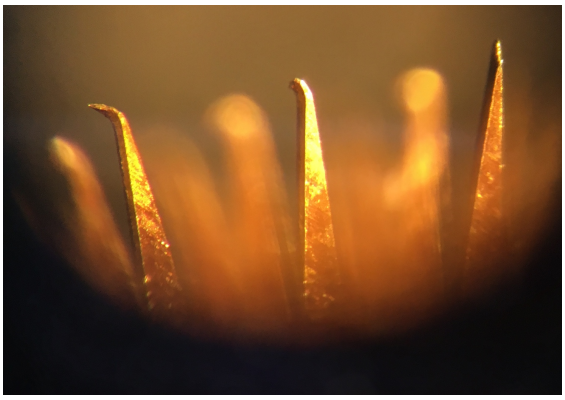
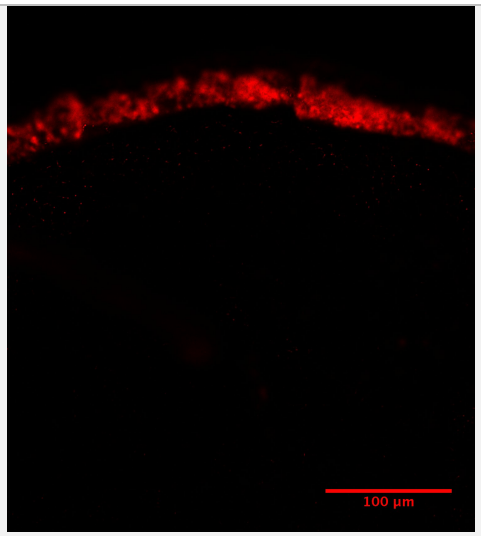
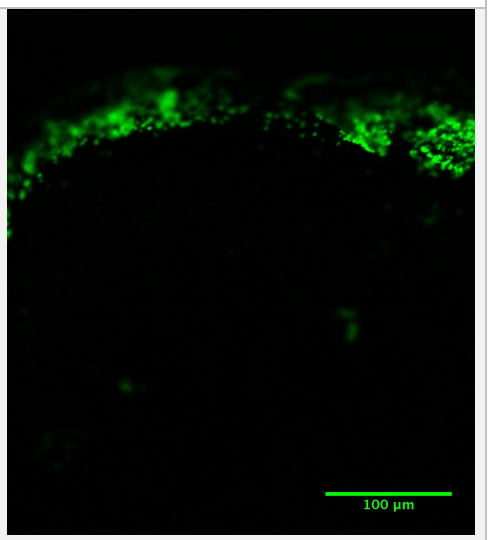
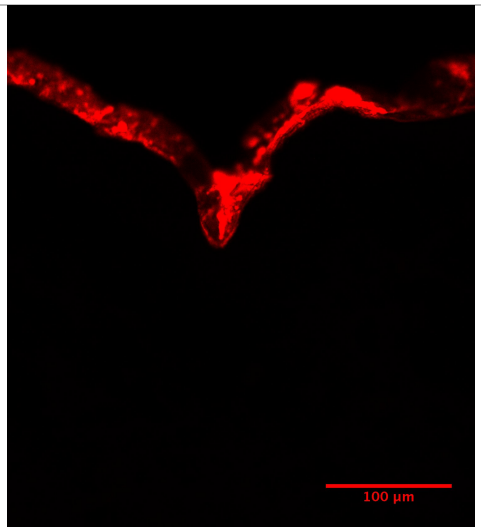
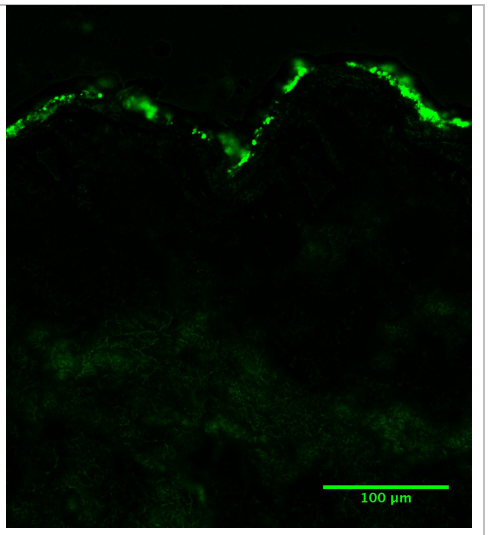
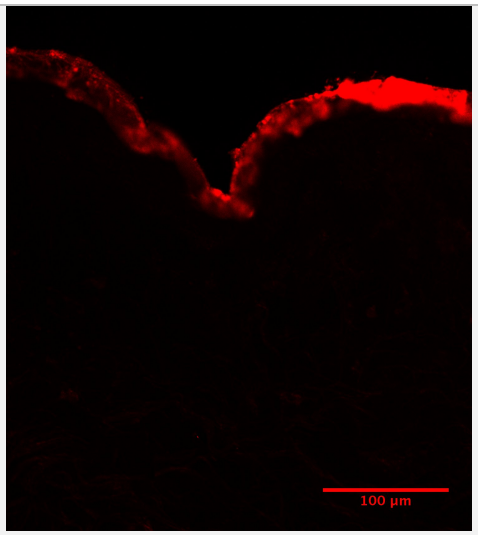
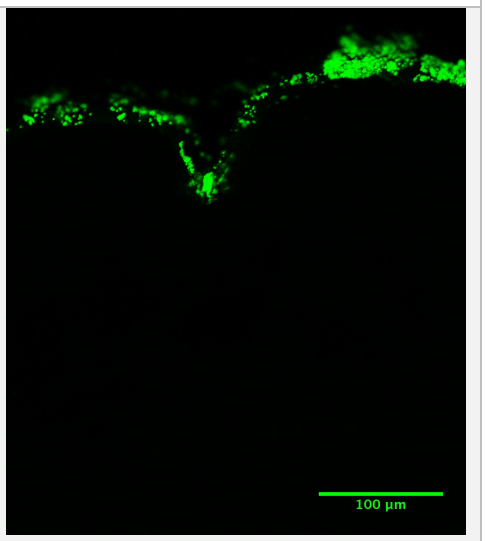
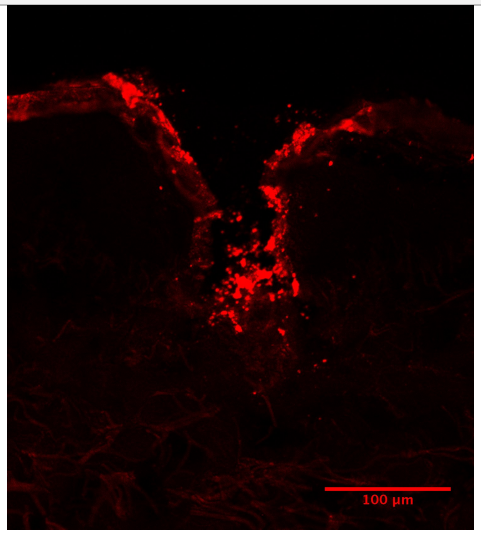
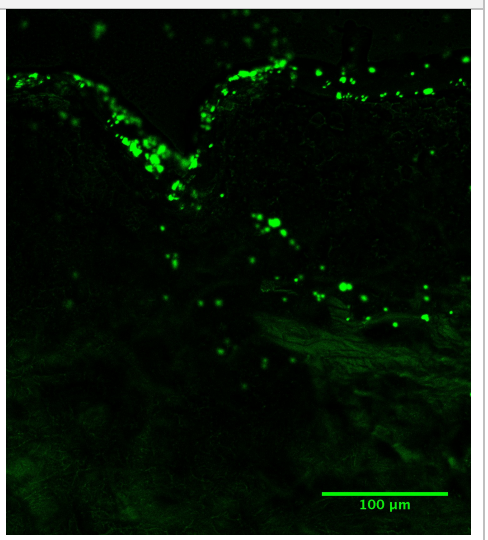


Figure 10 Varying needle sharpness. Picture obtained from the dermaroller used in this project after the dermaroller had been used three times on ten different skin samples with varying pressure.

4.2 Vertical penetration for dextran and beads

For every sample the vertical penetration through the skin was measured for beads down to a distance of 400 µm from the surface of the skin and a distance of 355 µm for dextran. Representative images of the five different samples pre-treated with 0 (control) 10, 20, 30 and 35 N can be seen in Figure 11, after topical application of the fluorophores.

	Dextran	**Beads**
Control		

10 N		
20 N		
30 N		

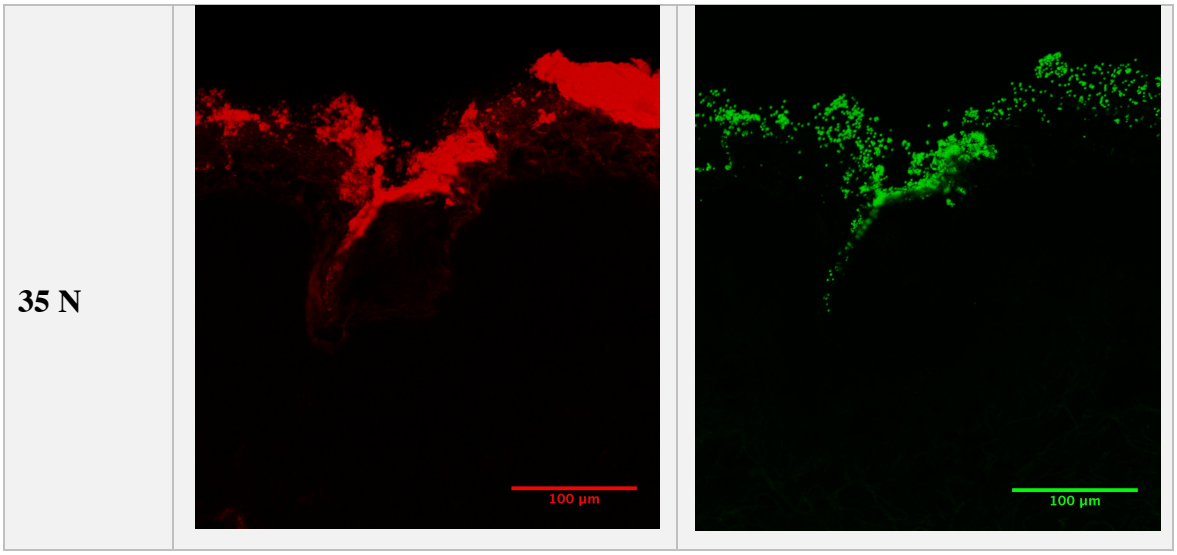


Figure 11 Representative images of skin samples treated with the dermaroller applied with different forces and afterwards treated with dextran and beads. Only impressions but no holes were observed for skin samples treated with a dermaroller with the pressure of 10 N and 20 N. However, penetration was seen for skin samples treated with a dermaroller with a force of 30 N and 35 N.

From Figure 11 the same tendency in distribution is seen for both dextran and beads. In the samples from both 10, 20, 30 and 35 N, the impressions/holes from the dermaroller can be observed. However, it should be noticed that no hole, only an impression is seen in the stratum corneum when the dermaroller is used with a force of 10 N and 20 N (Figure 11). Penetration of the stratum corneum is only clearly seen when the dermaroller was applied with forces of either 30 N or 35 N (Figure 11).

The thickness of stratum corneum as judged from the pictures in Figure 11 seems to vary as seen from the sample applied with either 10 N compared to the control. The stratum corneum seems to be thinner for the skin sample treated with 10 N compared to control. This is most likely due to the sample preparation when using the cryotome. The top of the stratum corneum was sometimes folded down, giving an impression of a thicker stratum corneum. Because it is the result of the preparation of the tissue samples after using the dermaroller, it has no impact on skin penetration of the needles of the dermaroller or the penetration of the test molecules.

4.2.1 Vertical penetration for dextran

4.2.1.1 Vertical penetration for dextran measured from the surface of stratum corneum

For dextran it was possible to determine the intensity in arbitrary units at different depth in the skin. Results are shown in Figure 12 with penetration depth along the x-axis and intensity in arbitrary units along the y-axis, where the measurements were started at the top of the stratum corneum. For

each column a standard error of the mean, SEM, was calculated.

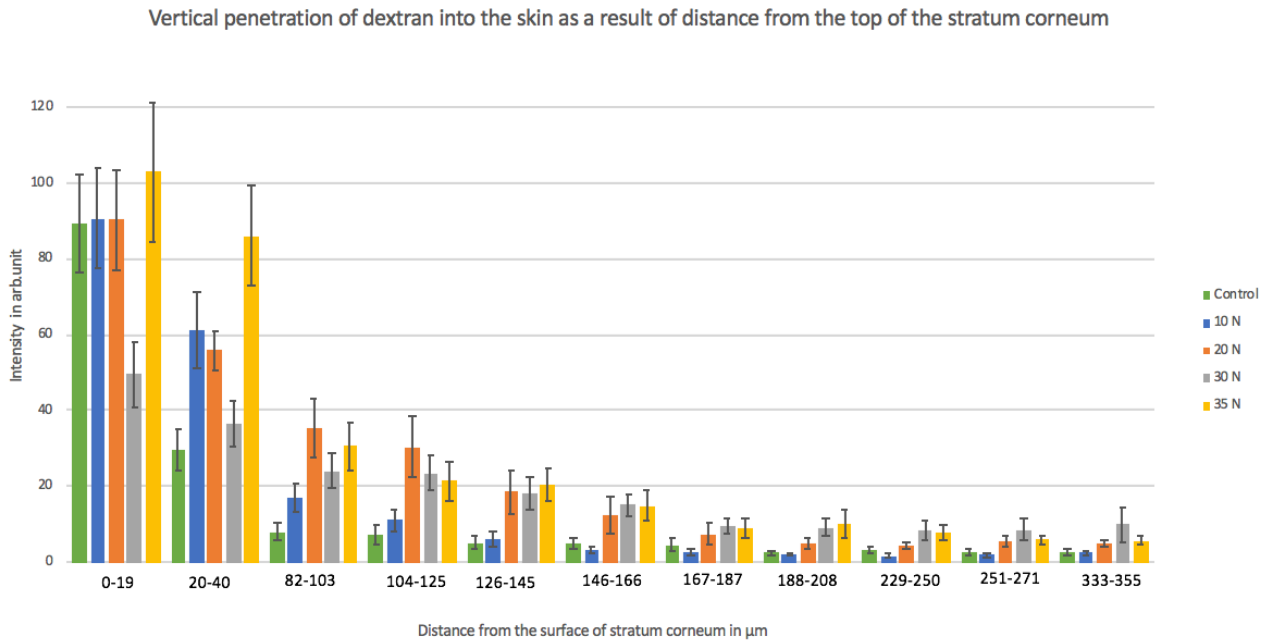


Figure 12 Intensity of dextran penetration measured as the result of the vertical depth starting at the surface of the stratum corneum. Intensity is depicted for each of the application forces (control, 10, 20, 30 and 35 N) used by the dermaroller. Penetration depth is depicted in µm along the x-axis, as the dextran intensity in arb.unit on the y-axis. Mean±SEM are shown, ($n_{control}$= 15, $n_{10\,N}$ = 18, $n_{20\,N}$= 16, $n_{30\,N}$= 17, $n_{35\,N}$= 13), n is the number of holes/impressions measured for each setting.

The high intensity in the interval from 0-19 µm corresponds to the thickness of the stratum corneum, because the measurements are started at the surface of the stratum corneum, and this may also be part of the explanation for the high intensity in the interval 20-40 µm. The intensity is expected to be highest at the top of the stratum corneum because this was where the test compound was applied.

The skin samples pre-treated with the dermaroller with a force of 30 N shows only half of the intensity compared to the other groups in the depth interval from 0-19 µm. This indicates that the fluorophores were not evenly distributed on the surface of the skin. Because of the uneven distribution of the test compound, the intensity values depicted in this graph have not been normalized, although this has been done in other experiments in this study (e.g. Figure 13). Uneven distribution of the test compounds has the effect that some values will be multiplied by a higher factor compared to others because the amount of test drug at the skin surface is low. If the values were normalized this could introduce an error of especially values obtained at the deeper layers of the skin where only autofluorescence is measured. Values only representing autofluorescence

would be multiplied by a higher factor in those samples with a low distribution of the test compound at the top of the stratum corneum. This could lead to a misinterpretation of the results and therefor no normalization was conducted.

The intensity of dextran is still high for skin samples treated with both 10, 20, 30 and 35 N even in the layers down to 125 μm. This does not necessarily mean that there has been penetration. In some cases, the stratum corneum was not perforated but instead bent downwards by the dermaroller, as seen in Figure 11 for both 10 N and 20 N.

For the skin sample treated with 20 N, penetration stopped at the interval of 188-208 μm. For both 30 N and 35 N intensities higher, than the control is still seen at a depth of 251-271 μm, some penetration might still occur afterwards, but our data are not accurate enough to make this conclusion. After 271 μm no distinct changes in intensities for the five skin samples are noticed.

4.2.1.2 Vertical penetration for dextran measured beneath the end of the impression/hole

Vertical penetration depth beneath the end of the impression/hole is also measured. Figure 13 shows how much of the dextran that reaches the epidermal and dermal layers of the skin. In this figure measures of stratum corneum is excluded in order to better depict the penetration into the skin by excluding the fluorophores staying only on the surface of the skin. This also avoids errors introduced because the dermaroller has made impression in the skin without penetration.

The depth of the starting points varies due to the varying depth of the impressions/holes (as also shown in Figure 9). It should be taken into account that the results are only based on five measurements of each sample. The intensities depicted at the y-axis in Figure 13 are normalized values. Normalization was conducted in order to compensate for local differences in the amount of dextran applied. The normalized intensity is measured based on the following equation.

$$Normalized\ intensity = \frac{intensity\ (arb.unit)}{max\ intensity\ (arb.unit)} \qquad \textit{Equation 1}$$

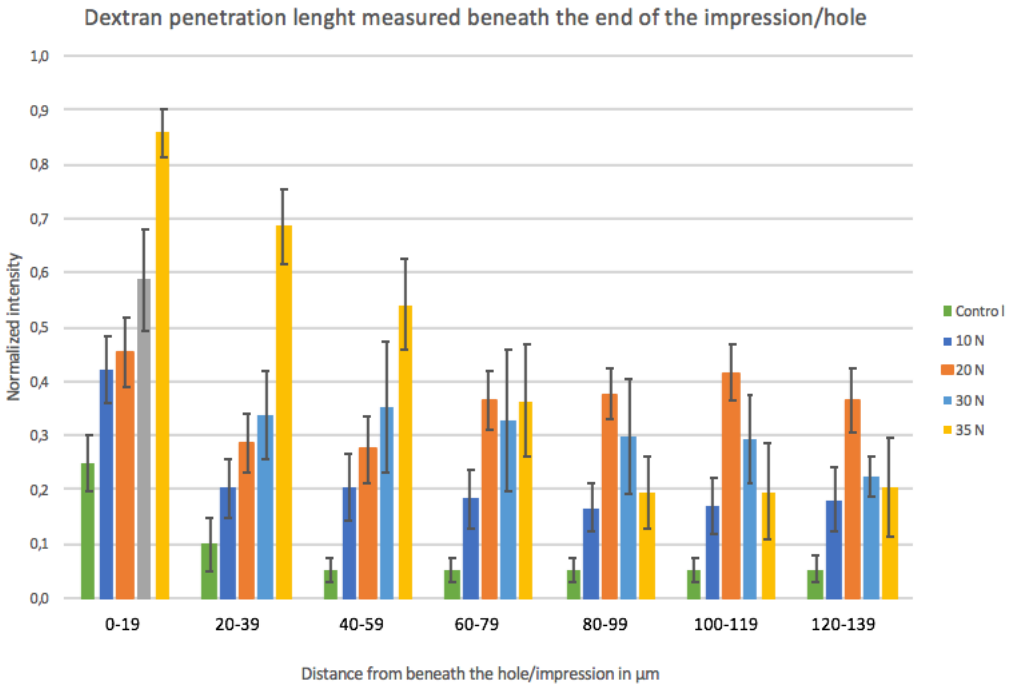


Figure 13 Dextran penetration into the skin. Penetration was measured starting beneath the impression/hole and moving through the epidermis into the dermal layer. The normalized intensity of dextran is depicted on the y-axis and the penetration depth in µm on the x-axis for the control, 10, 20, 30 and 35 N. Values are shown as mean±SEM (n=5).

As expected it is seen in Figure 13 that almost no penetration into the epidermis and into the dermal layers appeared in the control sample. The same is seen for the skin samples pre-treated with the dermaroller applied with 10 N. The normalized intensity for 10 N reaches a constant level fast. This is seen more easy in Appendix 4, Figure 20 where the same data have been depicted without setting up intervals for the depth in the skin. However, in both Figure 13 and Appendix 4, Figure 20, it is seen that intensity at 10 N is higher, but almost parallel with the control and it is possible that the explanation for this constant higher intensity in the skin samples treated with 10 N, compared to the control is due to autofluorescence.

In contrast, a high intensity is seen for both the skin samples treated with 20, 30 and 35 N. The skin sample treated with 35 N shows a huge penetration of dextran from 0-79 µm under the hole. Above 80 µm, the skin applied with 20, 30 and 35 N reaches a constant level in intensities. This is seen from 80 µm to 200 µm, where only small differences in intensity is seen for the five samples and the measured intensities most likely represented autofluorescence.

Figure 13 shows the mean±SEM for five different samples treated with a given force with the dermaroller. Variation between the five samples is seen and Figure 14 depicts the penetration of the

five individual samples that are pre-treated with a dermaroller applied with 30 N and included in Figure 13. As seen a great variation between the five different tissue samples is seen. Sample no. 5 decreases in intensity very fast and has a similar shape as the skin sample pre-treated with 10 N (shown in Figure 13 and in Appendix 4, Figure 20). This indicates that puncture of the stratum corneum leading to penetration of the dextran is not obtained in all samples. Sample no. 1 differs from the other four samples due to the high intensity and the fact that when all the other samples decreases in intensity sample no. 1 increases in intensity. Sample no. 3 demonstrated a slightly higher intensity than sample no. 5 which could indicate that the stratum corneum barrier is corrupted and that a small amount of dextran has penetrated the skin, although the penetration length is short. Taken together Figure 14 illustrates that 4 out of 5 samples show penetration of dextran when treated with a force of 30 N from the dermaroller, but the penetration length varies between the different samples.

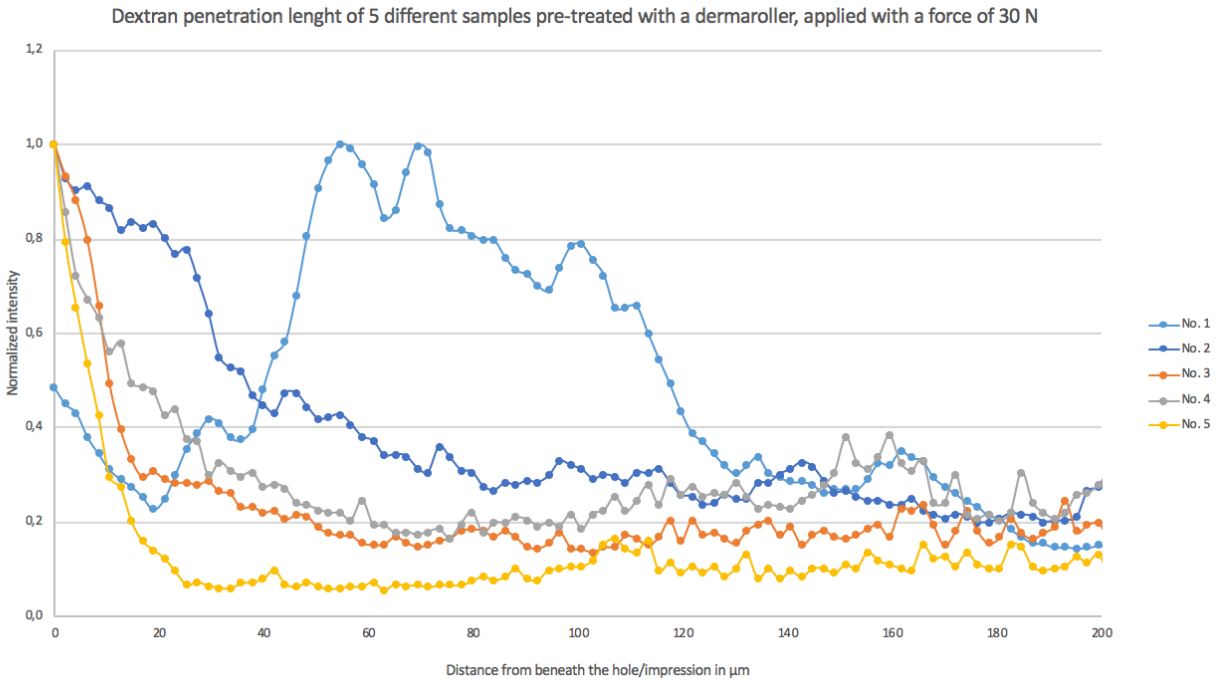


Figure 14 Dextran penetration depth of five different skin samples pre-treated with 30 N from the dermaroller and application of dextran. Penetration depth under the hole measured in µm on the x-axis and the normalized intensity on the y-axis.

Figure 15 is similar to Figure 14 except that it depicts the measurement of the penetration of the five samples that were pre-treated with a dermaroller applied with a force of 35 N. These samples are also included in Figure 13. For sample no. 2, 3 and 5 a minimal of intensity is reached after 90 µm, compared to sample no. 1 and 4. In sample no. 1 and 4 dextran penetrates as deep as 200 µm

which is the last measure point. A huge variation in both penetration length and amount of dextran is also seen for skin applied with a force of 35 N. All five skin samples show results indicating penetration of the stratum corneum.

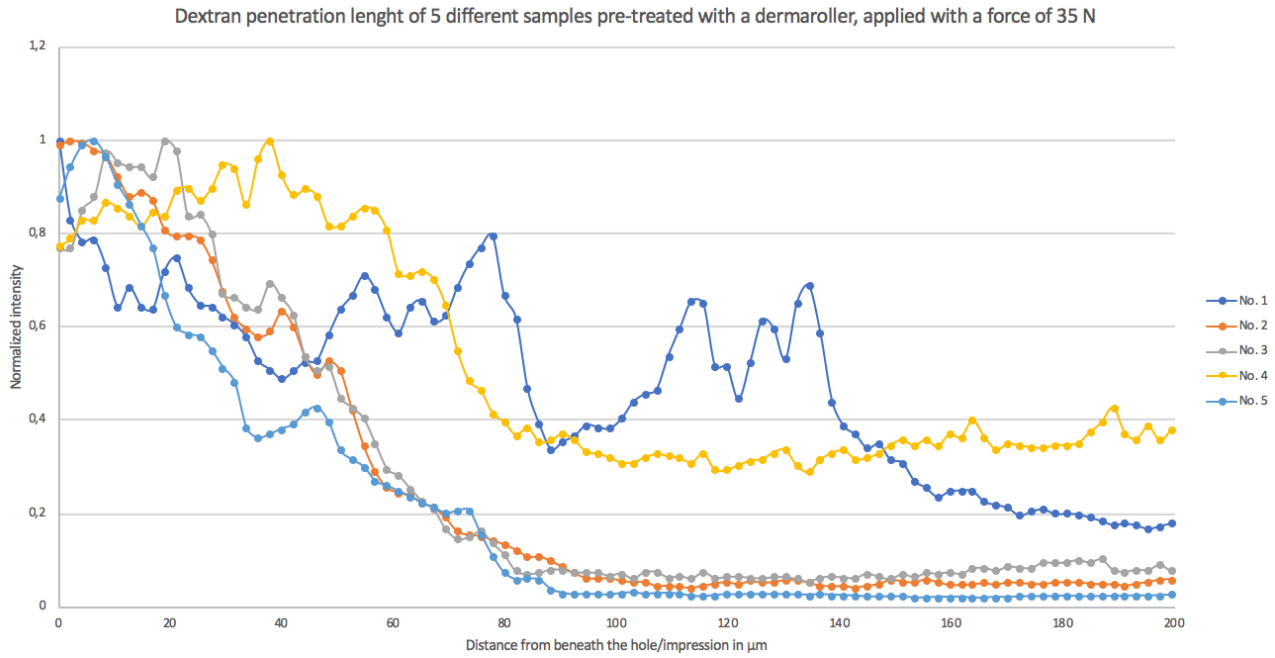


Figure 15 Dextran penetration depth of five different skin samples pre-treated with 35 N from the dermaroller and application of dextran. Penetration depth under the hole measured in µm on the x-axis and the normalized intensity on the y-axis.

Taken together the results obtained for vertical penetration of dextran showed that skin samples applied with a dermaroller with the force of 30 N and 35 N had the longest penetration length compared to skin samples treated as a control or applied with 10 N or 20 N from the dermaroller.

4.2.2 Vertical penetration of beads

Penetration of beads is also measured, and the results are shown in Figure 16.

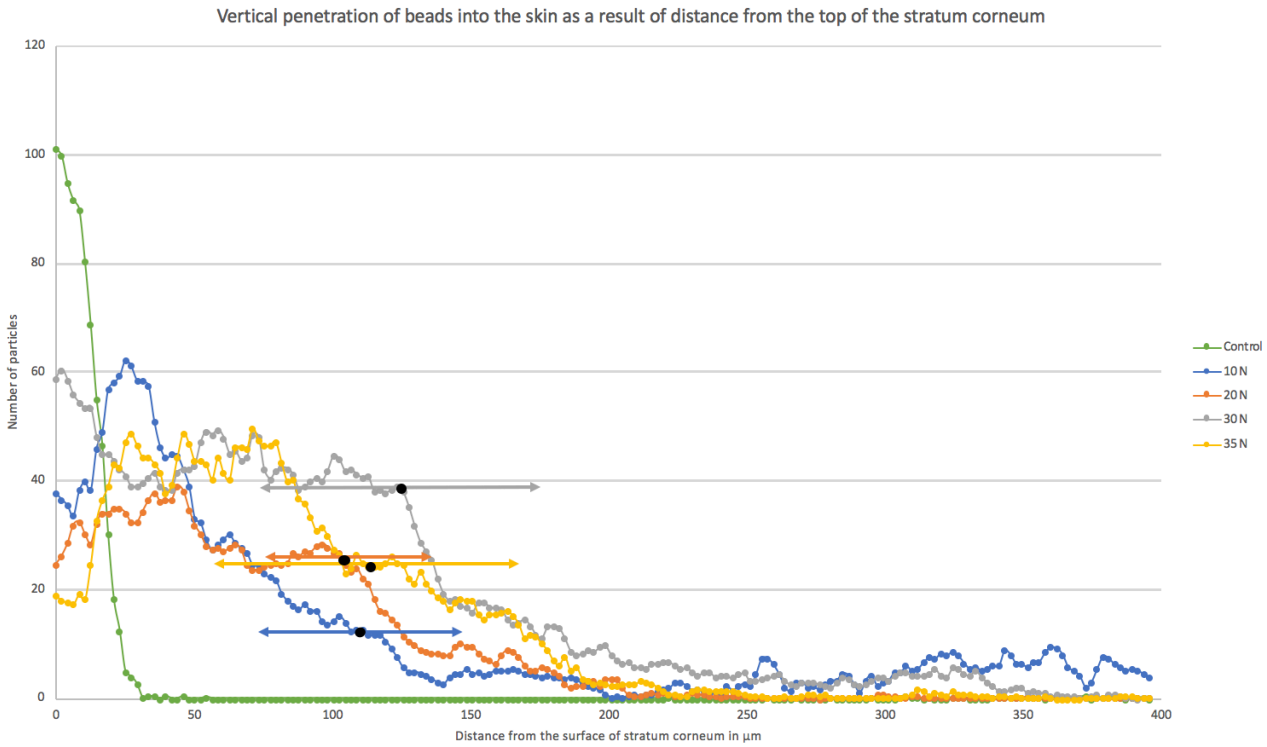


Figure 16 Beads penetration measured from the top of the stratum corneum. The correlation between vertical depth in µm on the x-axis and number of beads particles on the y-axis is shown. The graph shows data for control (n=5), and skin samples treated with 10 (n=20), 20 (n=20), 30 (n=21) and 35 N (n=13) from the dermaroller. The black dot is the mean value for the impression/hole depth measured in Figure 9 and the arrows corresponds to the SD value for the mean value (also obtained from Figure 9). It is seen that the beads in skin samples treated with 10 N are mainly confined to the bottom of the impression/hole, whereas the red, grey and yellow arrows show that beads penetration reached deeper in the skin also when including SD. The blue arrow indicates the SD value for the impression/hole depth when skin samples were treated with 10 N, red arrow corresponds to 20 N, grey arrow to 30 N and yellow arrow to 35 N.

The lower layer of the stratum corneum is reached where the graph of the control reaches almost zero in intensity and therefore that value represents the thickness of the stratum corneum. The starting point of the measurements is placed at the point where the maximum number of particles is reached for the control, which is at the top of the stratum corneum.

The graph of 10 N reaches zero faster than both 20, 30 and 35 N. The reason why it decreases slower than the control is most likely because the stratum corneum layer was bend downwards because of the force of the dermaroller. This trend is also seen for the rest of the samples. Results obtained with 10 N and measured beneath the mean hole depth (black dot on the blue line) ±SD

(blue arrow in Figure 16), shows either no or only very small amount of penetration (see Figure 16 and figure legend for details), or may be due to autofluorescence.

In contrast, it is clearly seen that skin samples pre-treated with 20, 30 and 35 N reaches almost zero particle numbers at a much deeper layer, 200-280 µm. Compared to the control samples and samples treated with only 10 N it is demonstrated that penetration of the test compound increases with increasing the force applied on the dermaroller, at least up to 35 N (Figure 16).

The results for the penetration depth of beads measured on five individual samples pre-treated with 30 N and 35 N are also obtained and can be seen in Appendix 5, Figure 21 and 22. Data reveal that for skin samples treated with 30 N, 2 out of 5 impressions/holes lead to puncturing and penetration of beads (Appendix 5, Figure 21). In skin samples treated with 35 N, 3 out of 5 impressions/holes lead to puncturing and thereby penetration of beads (Appendix 5, Figure 22). Compared to Figure 21, the number of particles, that has penetrated the epidermis and dermal layer, were lower in samples treated with a dermaroller with a force of 35 N (Figure 22 in Appendix 5, see absolute numbers on the y-axis).

In summary of the results obtained on vertical penetration of beads showed a penetration depth in the range of 200-280 µm in skin samples treated with a pressure of 20, 30 and 35 N from the dermaroller. However, the longest penetration depth was seen for skin samples treated with a dermaroller applied with 30 N.

4.3 Horizontal penetration of dextran

When measuring the horizontal penetration of dextran along the x-axis or along the stratum corneum barrier, the measurements started halfway through the impression/hole, as shown in Figure 8. Measurements continued 200 µm throughout the skin. This is done for 10, 20, 30 and 35 N.

The mean depth of the starting point for the horizontal measurement can be seen in Table 3. The depth is calculated as half of the total impressions/hole depth.

Table 3 Depth (µm) where the measurements of horizontal penetration started.

Batch	**Depth for measuring horizontal penetration, μm**
10 N	55.6
20 N	52.4
30 N	62.8
35 N	56.7

In Figure 17 the horizontal penetration length (measured in µm) of dextran is seen on the x-axis while the normalized intensity is seen on the y-axis. The normalized intensity is measured based upon Equation 1 shown above.

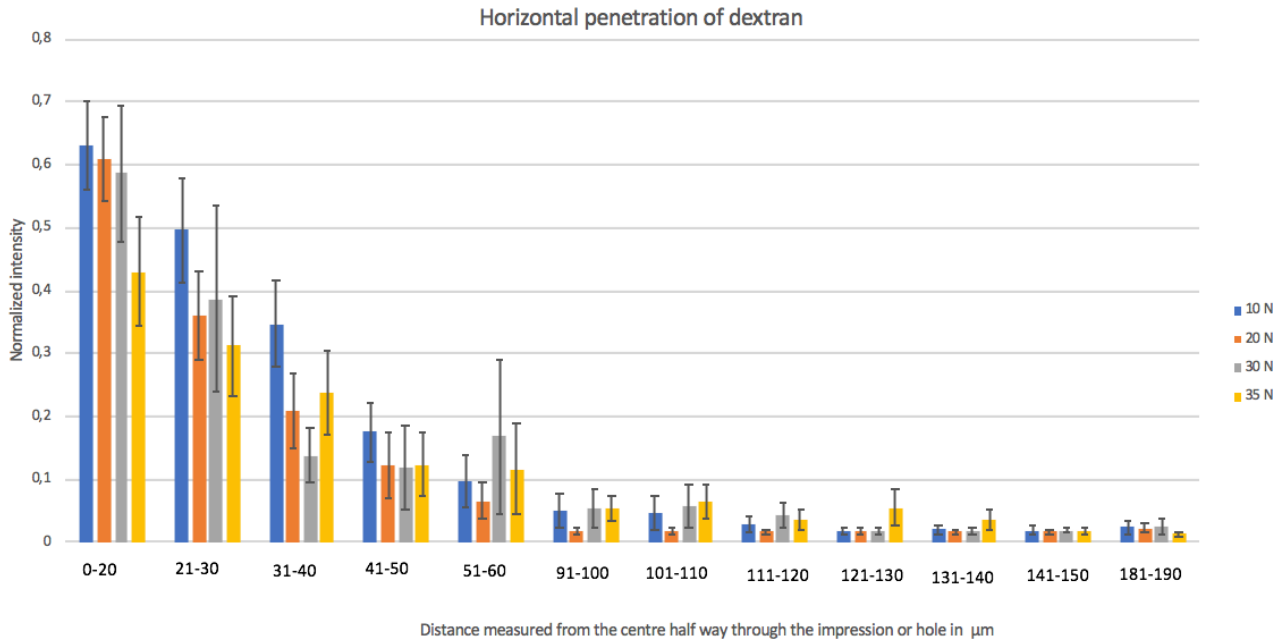


Figure 17 The intensity of the horizontal mean diffusion of dextran into the skin after pre-treatment of the skin with the dermaroller, applied with 10, 20, 30 and 35 N. Intensity of dextran shown on the y-axis was normalized, as seen from Equation 1. The intensities measured from 0-30 µm are the intensities measured on the stratum corneum. All the values are an average of the intensity with their ±SEM, ($n_{10\,N}$= 11, $n_{20\,N}$= 17, $n_{30\,N}$= 15, $n_{35\,N}$= 12).

The high intensity from 0-30 µm could correspond to the intensity from the stratum corneum because it is bended downwards with the impression or hole as seen in Figure 11. In Figure 17 a decrease in the intensity of the horizontal dextran distribution is seen with increasing distance from the centre of the impression/hole. That is the case for both 10, 20, 30 and 35 N. However, in the distance range between 121-140 µm it seems as there is a higher horizontal dextran penetration when pre-treating the skin with a dermaroller applied with 35 N compared to lower forces. Only measurements for horizontal diffusion of dextran was carried out.

5. Discussion

In this study the possibility of enhancing skin penetration depth and distribution of dextran and beads after pre-treating the skin with different prespecified forces with a dermaroller was investigated. Data showed that the dermaroller used with lower forces made only impressions in the stratum corneum whereas holes in the stratum corneum were made when used with higher forces and here penetration of the test molecules could occur. However, no correlation between the impression/hole depth compared to force applied to the dermaroller is seen, although all the forces used led to a deformation of the skin with a typical depth of around 115 µm. Horizontal penetration of dextran was obtained both when the skin samples were pre-treated with 10, 20, 30 and 35 N. For beads and dextran molecules, vertical penetration was observed. For dextran the maximum mean penetration length was 251-271 µm with 30 N and 35 N applied to the dermaroller. Likewise, for beads a maximum penetration length of 280 µm was obtained when 30 N was applied to the dermaroller.

5.1 Hole depth as the result of the force applied to the dermaroller

The intended use of the dermaroller is to puncture the stratum corneum barrier of the epidermis. By doing this it will be possible for nanoparticles to reach the deeper layers of the skin such as the remaining parts of the epidermis as well as the dermal layer. The epidermal and dermal layers have a thickness of 75 µm and 3-5 mm, respectively (1). From the data in Figure 9, it is seen that all the microchannels made as the result of the different forces applied to the dermaroller, have a greater hole depth or impression than 75 µm. This indicates that for those samples where the stratum corneum have been penetrated, the hole reaches the dermal layer, and delivering of drugs to this layer is therefore possible. However, it should be noticed that not all the dermaroller holes/impressions have led to penetration of the stratum corneum layer.

Based on results shown in Figure 9, no clear difference seems to be present between the hole depth created with the different forces tested on the dermaroller, because the SD's are overlapping. Instead it is seen that the use of a dermaroller with a force of either 10, 20, 30 or 35 N lead to a deformation of the skin with an average hole depth of 115 µm. Taken together no correlation between the force in newton applied to the dermaroller and the hole depth in µm was seen in this study.

In contrast to our study a previous study has shown a clear correlation where increasing the force, led to an increase in the hole size (26). There is no clear explanation for this discrepancy, but a possible explanation is differences in the sharpness of the needles on the dermaroller and elasticity of the skin. A previous study has shown the importance of a sharp needle. The authors concluded that a sharper needle led to a reduction in the force needed to puncture the skin (27). The needles used in this project were blunt when used only a few times, (Figure 10). This could have had a great impact on the hole depth and also serve as an explanation for the variation in the results obtained in this study. It is possible that the needles used at the beginning of the project demanded a lower force to obtain the same results as the blunt needles in the end of the project. Our data has not yet been analysed to clarify this but from a theoretical standpoint it could serve as an explanation. Studies have also shown that the viscoelastic property of the skin have an effect on the hole size. Elasticity of the skin is therefore an important feature to the possibility of puncturing the skin barrier. It varies compared to both sex, age and site of the body (28-30). These variations make it difficult to obtain a standardized method and compare the results in relation to microneedle penetration, because of the different resistance provided by the skin.

Another study has also shown that microneedles with a length of 770 μm created microchannels with an average depth of 152.5±9.6 μm (31). However, in this study the force applied to the microneedles was not mentioned, and it is therefore difficult to compare their results with the results obtained in this study. However, it underscores the impotence of our project, especially when looking at the fact that the amount and the penetration length of the fluorophores varies according to the force applied to the skin surface with the dermaroller. The study by Kalluri et al. also studied the closure time for the pores made by the dermaroller. These results indicated that after 18 hours pores were closed when microneedles with a length of 770 μm were used (31). Our skin samples were collected and studied after 22 hours, which could have led to a reduction of the hole size, giving an explanation to the smaller hole size found in our study.

In this project the force applied by the dermaroller was done by hand whereas other studies have used a device for insertion of microneedles (27, 30). The manual use of the dermaroller gives a larger variation in the pressure applied to the skin, when the dermaroller roles over the skin barrier. Likewise, the velocity of the dermaroller also varied from skin sample to skin sample and could have an impact on the hole size and penetration. To overcome this problem and to standardize the hole size and thereby the penetration length according to different forces applied, the force to the needles could be made electronic. This means that the force from the dermaroller should not be

done by the patient itself, but instead by the dermaroller. This would most likely give a more reproducible force and a more uniform hole, which would lead to more consistent delivering of drug through the skin, which is important if the dermaroller is to be used in practise (30, 31).

5.2 Vertical penetration of dextran and beads

As mentioned before molecules with a weight of less than 500 Da are more likely to passively penetrate the skin barrier. In this particular project dextran molecules were used with a molecular weight of 70,000 Da. They are categorised as a macromolecule, and thereby having a molecular weight much larger than 500 Da. The penetration of dextran is therefore not expected to occur unless forced enhancement is applied. Beads are categorized as nanoparticles and as for dextran no penetration is excepted unless the stratum corneum barrier is punctured or disrupted in other ways.
Data obtained for dextran penetration showed that the greatest penetration was seen for skin samples pre-treated with 30 N, although the difference from 35 N was small (see Figure 12). Both 30 N and 35 N showed a penetration depth of 251-271 μm measured from the top of the skin barrier and maybe even further, but our data were not sensitive enough to determine that. However, samples treated with 35 N showed that more holes led to a penetration of dextran (punctured skin), compared to application of 30 N (see Figure 14 and 15). The counting of penetration holes has only been made on five samples, but more reliable data could be obtained if further investigation with a large number of samples were included.
Inconsistent results regarding dextran penetration were obtained in the measuring of skin samples pre-treated with 20 N. However, comparing with the images from skin samples treated with a dermaroller with 20 N indicated that almost nothing penetrated the stratum corneum barrier. This lead to the conclusion that a force of 20 N in rare occasion would lead to a penetration of dextran, but it makes it impossible to standardize the use of the dermaroller if only a force of 20 N is applied.
Data on penetration of beads showed that 30 N gave the largest penetration length for beads, also when compared to pre-treatment with 35 N (Figure 16). Comparing these results with the frequency of puncturing the stratum corneum layer, the skin samples applied with a force of 35 N by the dermaroller led to a higher frequency in puncture of the stratum corneum compared to 30 N (Appendix 5 Figure 21 and 22). However, as mentioned previously measurements below the holes, were only obtained from five impressions/holes, and further investigation is certainly needed.

Only a limited number of studies have been made on nanoparticle penetration length through microchannels. In one study by Zhang et al. the mean sizes of the nanoparticles used were, 160.1, 205.5 and 288.2 nm (32). For comparison, in our project the dextran molecules were 20 nm and beads molecules were 1000 nm. The study by Zhang et al. showed that no nanoparticles were able to penetrate the stratum corneum barrier when no microneedles were used. However, the use of microneedles led to the deposition of nanoparticles in epidermis and a smaller amount in the dermal layer, but the penetration through the microchannels were size-dependent. Smaller nanoparticles showed a greater ability to penetrate the skin layers (32). These findings correspond to the observation made in this project, both dextran and beads molecules stayed in the epidermis and dermis layer, although the penetration length for dextran and beads was very much alike in our study, despite the size difference.

Overall our results showed that skin samples pre-treated with a dermaroller applied with a force of 30 N and 35 N led to the largest penetration length of dextran and skin samples treated with 30 N led to the largest penetration length of beads. Although skin samples treated with 35 N led to a higher number of holes in the stratum corneum. But the difference in using 30 N or 35 N was only small and not as pronounced as the difference between 20 N or 30 N.

5.3 Horizontal penetration of dextran

Data from this study showed that enhanced horizontal penetration is possible when a dermaroller is used. The high intensity measured in Figure 17 in interval 0-30 µm most likely corresponds to the thickness of the stratum corneum, because it is known that stratum corneum varies in thickness between 10-30 µm (11). Skin samples applied with 35 N shows the greatest penetration of dextran with a depth interval of 131-140 µm. Surprisingly penetration of the skin samples treated with 10 N and 20 N also occurred, with 10 N as the one with the longest penetration. There is no good explanation for the finding that a lower pressure at 10 N leads to a larger penetration depth than 20 N and therefor further studies are needed.

The vertical depth of all the measurements were started in the interval of 52-62 µm, which corelates with the depth of epidermis. Therefore, when looking at horizontal penetration, delivering of drugs to the epidermis seems to be possible.

In conclusion horizontal penetration is possible, when the skin has been pre-treated with the use of microneedles applied with a force of 10, 20, 30 and 35 N, but the longest penetration is seen when the skin was treated with 35 N.

Little research on horizontal penetration of test drugs when using a dermaroller has been conducted, although one other study has analysed horizontal penetration of the drug, lidocaine (33). Lidocaine is a smaller molecule, with a molecular weight of less than 500 Da and it can therefore diffuse passively. However, their results are consistent with the results from this study and they conclude that permeation of lidocaine is increased when using microneedles, although no correlation between applied force and amount of penetrated lidocaine was observed (33).

6. Conclusion

The aim of this study, was to investigate the ability to puncture the stratum corneum layer and increase the penetration of drug-analogues as a consequence of applying different forces to the dermaroller. Our hypothesis was that within a specific force range multiple holes in the stratum corneum would appear leading to an optimal skin penetration.

Horizontal penetration for dextran was observed for skin samples applied with both 10, 20, 30 and 35 N but the longest penetration length at 131-140 µm was observed for 35 N.

The vertical penetration length of dextran showed that penetration happened when skin was pre-treated with 30 N, and the dextran reached a depth of 251-271 µm, measured from the surface of the stratum corneum. For beads nanoparticles the longest penetration depth measured from the top of the stratum corneum was seen in the skin sample treated with 30 N with a depth of 280 µm.

Taken together, to insure penetration a force of 30-35 N applied by the dermaroller were needed. The largest penetration length and the highest frequency of penetrated holes were obtained with forces in the higher end of our test spectrum namely 30 N and 35 N. This indicates that the force applied to the dermaroller is important for drug delivery, and further studies in this method may in the future pave the way, to modulate drug penetration into the skin. However, a standardized method for applying the force must be used to insure a consistent penetration enhancement and dosage. Further studies are needed with more skin samples, new dermarollers and if possible an even more standardized way to apply the precise force of the dermaroller.

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9. Appendix

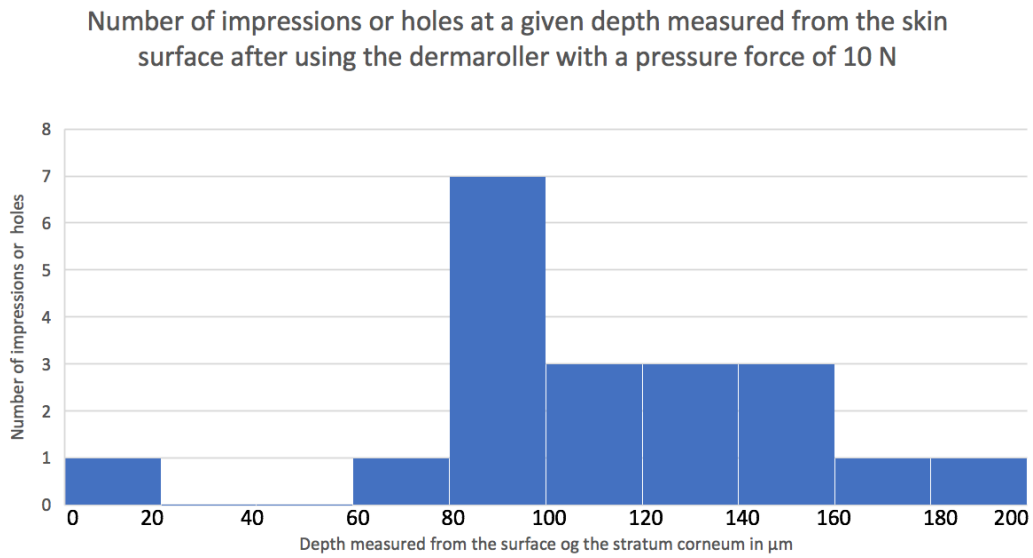
9.1 Appendix 1

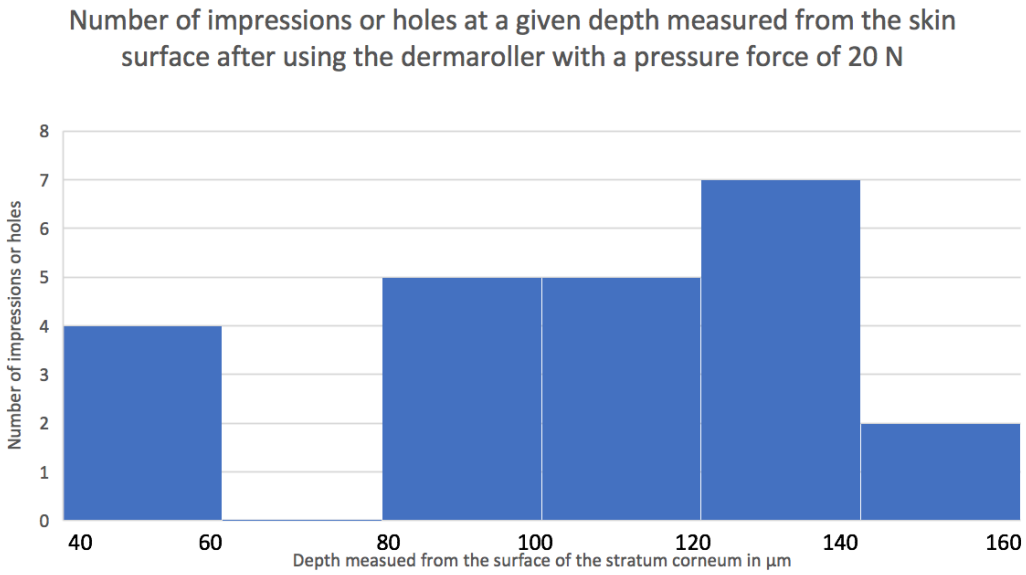
Table 2 Mean±SD for the force applied with the dermaroller, n=2.

Intended force, *N*	**Mean force value, *N***	**SD, *N***
10 N	10.38	0.48
20 N	19.67	0.94
30 N	28.83	1.57
35 N	34.08	0.61

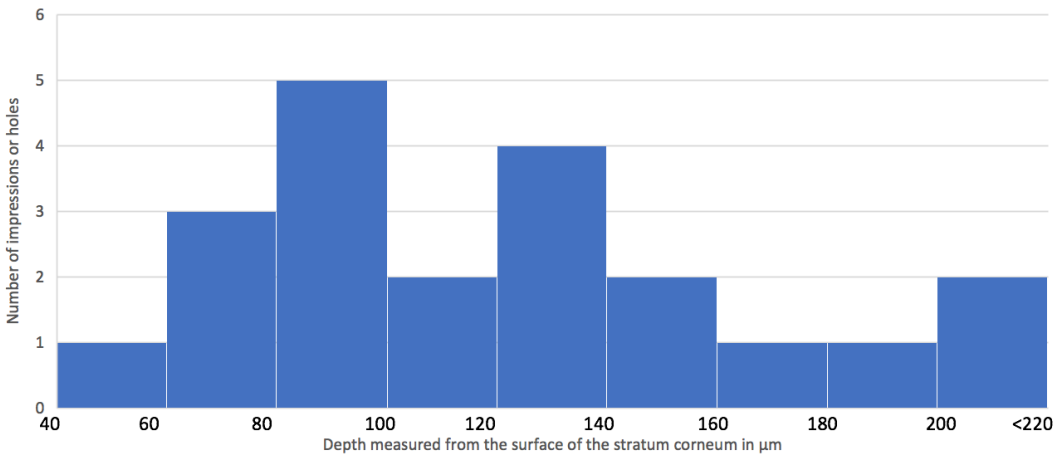
9.2 Appendix 2

Figure 18 Histograms of variation in hole depth in relation to applied pressure with the dermaroller. On the x-axis is the depth in µm from the skin surface and on the y-axis are numbers of holes/impressions. Each histogram represents a pressure by the dermaroller of either 10 N (n=20), 20 N (n=23), 30 N (n=21) or 35 N (n=14), n is the number of impressions or holes for each force tested.

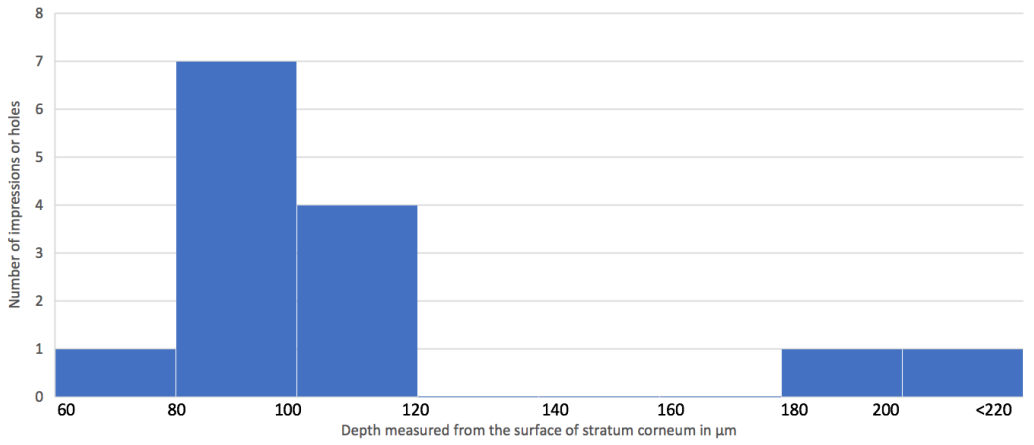




Number of impressions or holes at a given depth measured from the skin surface after using the dermaroller with a pressure force of 30 N



Number of impressions or holes at a given depth measured from the skin surface after using the dermaroller with a pressure force of 35 N



9.3 Appendix 3

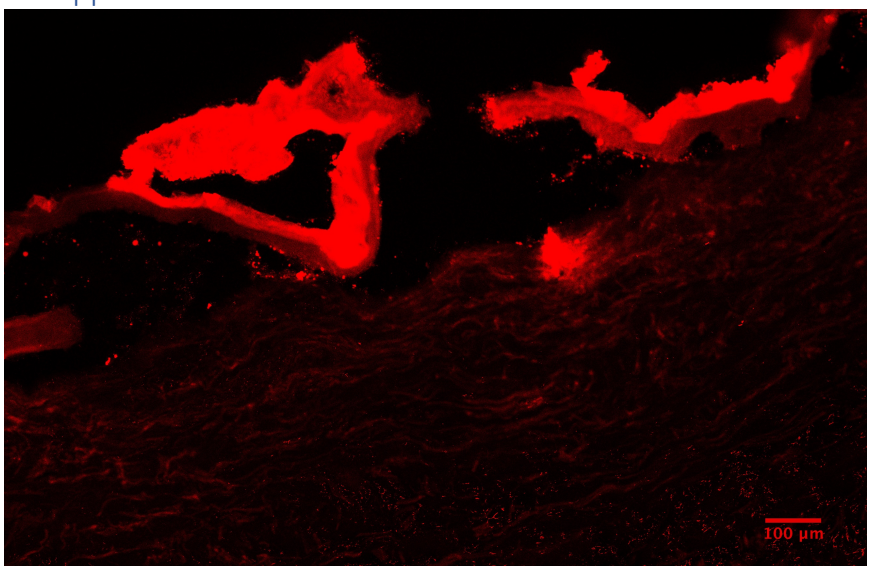


Figure 19 An image of the destroyed stratum corneum after the skin sample was treated with the dermaroller applied with 35 N. The red colour is obtained from dextran. the dextran only stays in the stratum corneum. Corruption of stratum corneum only appeared at the first batch.

9.4 Appendix 4

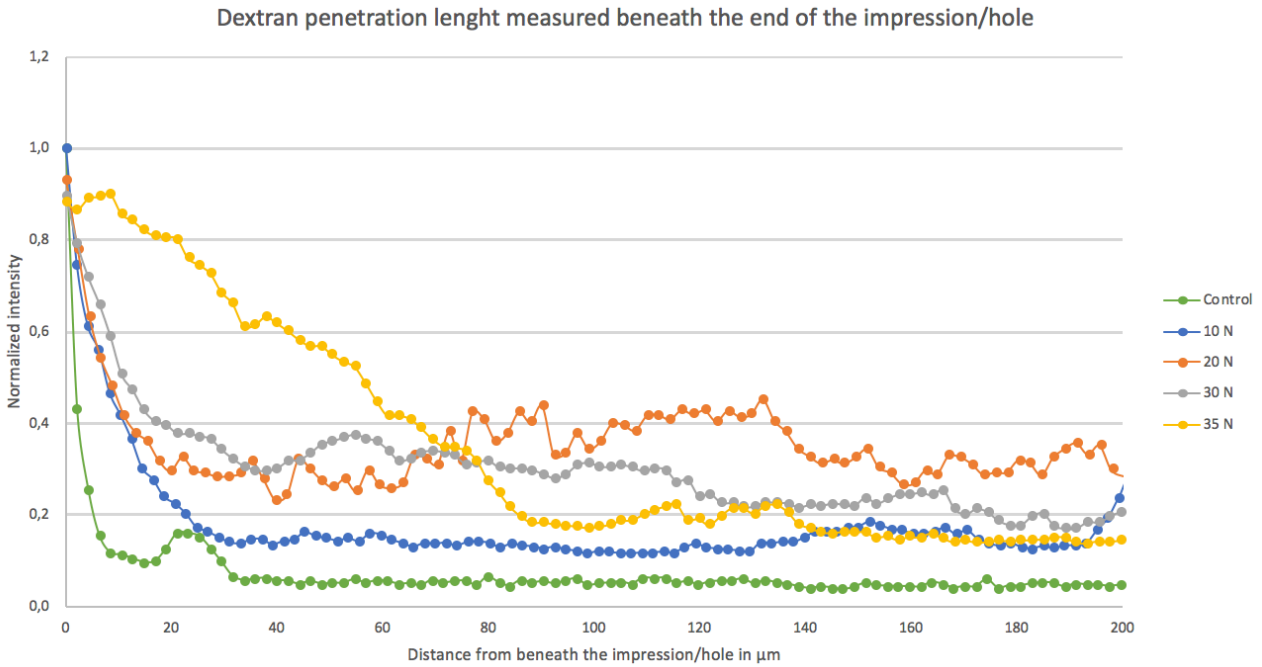


Figure 20 shows the depth in µm on the x-axis, with the normalized intensity on the y-axis. Skin samples were treated with dextran and the respective forces, 10, 20, 30 and 35 N and one as a control, n=5.

The graph is based on the same data as for Figure 13. The graph is made so comparison of data from Figure 14 and 15 is easier.

9.5 Appendix 5

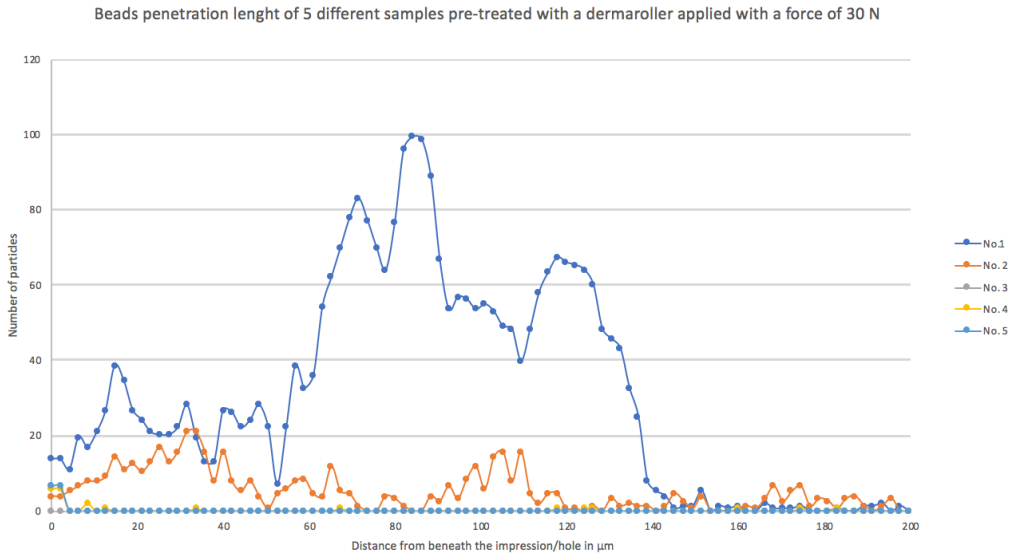


Figure 21 depicts the measurement of beads in the five samples that were pre-treated with a dermaroller applied with a force of 30 N. Penetration depth started under the impression/hole was measured in µm and shown on the x-axis and number of particles is shown on the y-axis. Large variation in the five measurements was seen. Only 2 out of 5 measurements indicate that penetration had happened, no. 1 and 2. For these 2 samples the variation in the number of particles penetrated into the epidermis and dermal layers is huge.

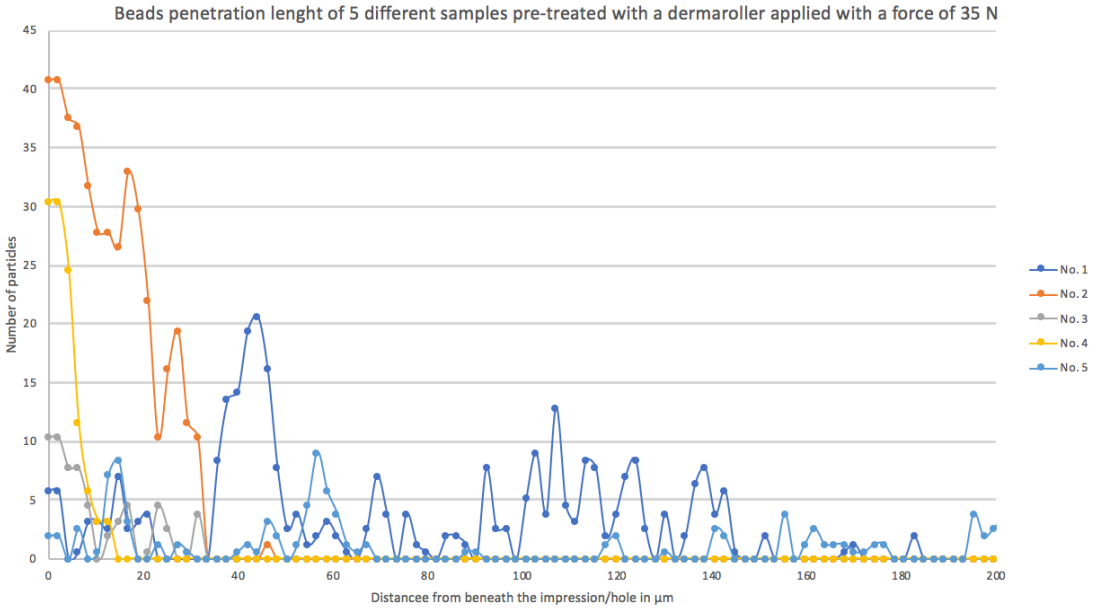


Figure 22 depicts the penetration of beads in the five samples that were pre-treated with a dermaroller applied with a force of 35 N. Penetration depth started under the impression/hole was measured in µm and shown on the x-axis and number of particles on the y-axis. 3 (sample 1, 2 and 5) out of 5 samples showed evidence of penetration.