Monitoring the penetration process of single microneedles with varying tip diameters

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ABSTRACT

Microneedles represent promising tools for delivery of drugs to the skin. However, before these microneedles can be used in clinical practice, it is essential to understand the process of skin penetration by these microneedles. The present study was designed to monitor both penetration depth and force of single solid microneedles with various tip diameters ranging from 5 to 37 μm to provide insight into the penetration process into the skin of these sharp microneedles. To determine the microneedle penetration depth, single microneedles were inserted in human ex vivo skin while monitoring the surface of the skin. Simultaneously, the force on the microneedles was measured. The average penetration depth at 1.5 mm displacement was similar for all tip diameters. However, the process of penetration depth was significantly different for the various microneedles. Microneedles with a tip diameter of 5 μm were smoothly inserted into the skin, while the penetration depth of microneedles with a larger tip diameter suddenly increased after initial superficial penetration. In addition, the force at insertion (defined as the force at a sudden decrease in measured force) linearly increased with tip diameter ranging from 20 to 167 mN. The force drop at insertion was associated with a measured penetration depth of approximately 160 μm for all tip diameters, suggesting that the drop in force was due to the penetration of a deeper skin layer. This study showed that sharp microneedles are essential to insert microneedles in a well-controlled way to a desired depth.

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1. Introduction

The skin provides an important physical barrier to the environment. It is rich in immune-responsive cells, such as Langerhans cells and dermal dendritic cells. Consequently, it provides a promising location for vaccination. By administering the vaccine directly to the epidermal and dermal layers of the skin, a lower dose of antigens may be required to achieve a comparable immune response to subcutaneous or intramuscular vaccination with a hypodermic needle (Chen et al., 2010, 2011; Fernando et al., 2010; Gelinck et al., 2009; Quan et al., 2010). Vaccination with microneedles represents a promising method to deliver antigens into the skin. These projections with a length of less than a millimetre can be fabricated from various materials in a wide range of geometries (Donnelly et al., 2010a; Kim et al., 2012; van der Maaden et al., 2012). Microneedles are used in multiple ways. For example, hollow microneedles can be used to inject fluids into the skin. By contrast, solid microneedles can either create micropores in the stratum corneum after which drugs can be topically applied (poke and patch) or they can release the vaccine into the skin after their insertion. Using this last approach two methods are in development, coated microneedles and dissolvable microneedles.

An important challenge in using microneedles to deliver vaccines into the skin in a reproducible manner is to effectively and reproducibly penetrate the skin. Therefore, microneedle penetration of the skin has to be fully characterised, including the inter-subject variation in skin mechanical properties and the various geometries of the microneedles. It is of paramount importance to obtain sufficient control on the depth of penetration of the microneedle into the skin since only the part of the microneedle within the skin will deliver the drug. In addition, the penetration depth may be important to specifically target Langerhans cells or dermal dendritic cells residing in the epidermal and dermal layer of the skin, respectively. Activation of these different cells may result in a different immune response (Banchereau et al., 2009; Romani et al., 2010).

During microneedle application, the skin is first indented before penetration occurs. As a result, many studies reveal that the insertion depth of microneedle arrays range widely from 10 to 80% of the microneedle length (Chu and Prausnitz, 2011; Coulman et al., 2010; Crichton et al., 2010; Donnelly et al., 2010b; Kalluri et al., 2011; Lee et al., 2008; Li et al., 2009; Matriano et al., 2002; Roxhed et al., 2007). Various factors affecting microneedle penetration depth have been examined. The penetration depth of a microneedle array increases with application velocity (Crichton et al., 2010) and application force (Donnelly et al., 2010b). Microneedle length also influenced the penetration depth, in contrast to microneedle interspacing. Accordingly, most studies have employed microneedles in an array, with a high velocity or large force to ensure effective penetration of the skin. However, the penetration mechanism of a single microneedle is still yet to be fully clarified.

Clearly the application force is critical. A previous study reported that insertion force of relative blunt microneedles, with tip diameters ranging from 60 to 160 μm, ranged from 0.08 to 3.04 N and was linearly dependent on tip frontal area (Davis et al., 2004). These relatively high insertion forces motivated the design of sharper microneedles, with a resultant reduction of insertion force measured for microneedle arrays (Khanna et al., 2010; O’Mahony, 2014; Roxhed et al., 2007).

Evidently, penetration depth as well as penetration force are important factors in controlled application of microneedles. However, the mechanism of microneedle penetration and the relation between penetration force and depth are still not well understood. Therefore, the present study was designed to monitor both penetration force and depth of single microneedles, manufactured to a range of tip diameters. In particular, it was designed to examine the influence of tip diameters on the nature of the penetration mechanisms and the absolute values of the penetration parameters.

2. Materials and methods

2.1. Microneedle fabrication

Single solid microneedles were fabricated in a controlled manner by pulling glass rods with a diameter of 1 mm (World Precision Instruments, Inc, Sarasota, FL, USA) with a micropipette puller (P-97, Sutter Instruments Company, Novato, CA, USA) (Martanto et al., 2006a). Microneedles varying systematically in tip diameter were produced by tuning the input parameters of the micropipette puller. In addition, in some cases the microneedle was cut to the desired tip diameter using a microforge (MF-830, Narishige, Japan). Four sizes of microneedles were produced with tip diameters of 5, 15, 24, and 37 μm with a resolution of 1 μm. For each microneedle, an image was made with a digital microscope to determine its shape (Fig. 1). The tip angle between differently sized microneedles varied from 11 to 21 degrees. The tip length of the microneedles was approximately 3.3 mm.

2.2. Preparation of human skin samples

Abdominal human skin was obtained from 8 female patients, aged between 35 and 55 years, from the Catharina Hospital, Eindhoven, the Netherlands, according to Dutch guidelines of secondary used materials. The skin was transferred to the laboratory and processed within 4 h of excision. For processing, the skin was stretched on a stainless steel plate, using multiple forceps, and cleaned with ethanol (Geerligs et al., 2011a; Lamers et al., 2013). Subsequently, skin slices of 1.20 ± 0.23 mm thickness (mean ± standard deviation), which contained the stratum corneum, viable epidermis, and dermis, were obtained using a dermatome (D42, Humecta, Enschede, the Netherlands). These slices were cut into squares of approximately 3 by 3 cm. Thereafter, speckles of Graphit 33 were sprayed on the skin samples to increase contrast of features during imaging.

Natural pretension in the skin leads to shrinkage during the cutting process. As the penetration can be influenced by the tensions within the skin samples (Butz et al., 2012), the in vivo state should be mimicked as closely as possible. This was achieved by attaching a metal ring of 7.5 g weight with an inner diameter of 15 mm on the unstretched skin samples.
with super glue (Ultra gel, Pattex). Subsequently, the skin sample was stretched by the weight when it was placed on a rod with a smaller diameter than the ring. A second metal ring with an inner diameter of 4.4 mm was then glued on top of the stretched skin. Using digital image correlation, pilot tests indicated that the skin sample is stretched between 15 and 25%, considered to represent the skin strains encountered in vivo. During and after sample preparation, the skin samples were stored in Hank’s Balanced Salt Solution (HBSS, Lonza Biowhittaker, Switzerland), supplemented with 1% penicillin/streptavidin and 0.2% Fungin at 4°C until further use. All experiments were performed within 48 h of surgery.

### 2.3. Microneedle penetration of the human skin

The microneedles were attached to a custom designed micro-indentation device (Vaenkatesan et al., 2006). With this device, displacement of the microneedle can be applied. Under optimal conditions, the displacement and force can be measured with a resolution of 15 nm and 15 μN, respectively.

Skin samples were allowed to warm up to room temperature. Subsequently, the samples were taken out of the HBSS. The larger metal ring and excessive skin around the smaller ring were removed. Any excess fluid was removed from its top surface, and the sample, with the small metal ring still attached, was placed on a substrate of polydimethylsiloxane (PDMS). The sample was positioned in such a way that the microneedle was located above the centre of the smaller metal ring attached to the skin surface.

Microneedles were indented to a predetermined maximal displacement at a velocity of 0.05 mm/s. This velocity assured quasi-static conditions and reliable force data. When the predetermined maximal displacement was reached, the microneedles were immediately retracted at the same rate until the tip was positioned 500 μm above the skin surface. During microneedle indentation, the surface of the skin was continuously imaged from an angle of approximately 55 degrees with a monochromatic CCD camera (DMK 41BU02.H, The Imaging Source, Germany), which was attached to a long distance microscope (QM100, Questar, New Hope, PA, USA) (Fig. 2). This resulted in a series of video frames and force data for each experiment.

Several series of experiments were conducted. One series of experiments focussed on the influence of the skin donor on the penetration depth. These experiments were conducted with microneedles with a tip diameter of 5 μm using samples of 5 different skin donors. A second series of experiments focussed on the effect of the tip diameter on the resulting penetration depth and force. Tip diameters varied between 5 and 37 μm. Experiments of both series were also used to evaluate the penetration depth measurements. A summary of the different experiments is provided in Table 1.

**Fig. 1** – Solid microneedles with a tip diameter of 5, 15, 24, and 37 μm. The microneedles were fabricated using a micropipette puller. Bar indicates 50 μm.

**Fig. 2** – The surface of the skin was recorded to determine the penetration depth of the microneedle. (A) Video frame used to measure the width of the microneedle at the location where the surface of the skin was penetrated (white bar). (B) Schematic overview of the setup. From the measured width, the penetration depth was deduced, knowing the shape of the microneedle.
Table 1 – Summary of the experiments performed to determine the influence of the used skin donor and the tip diameter. Experiments of both series were also used to evaluate the protocol of the penetration depth measurements.

<table>
<thead>
<tr>
<th>Type of test</th>
<th>Microneedle tip diameter [μm]</th>
<th>Maximal displacement of microneedle [mm]</th>
<th>Number of samples</th>
<th>Number of skin donors</th>
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<td>8</td>
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<tr>
<td>measurements</td>
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<td>0.25</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>0.50</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>1.00</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Influence of skin donor on penetration depth</td>
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<td>0.25, 0.50, 0.75, 1.00, 1.25, and 1.50</td>
<td>33</td>
<td>5</td>
</tr>
<tr>
<td>Influence of tip diameter on penetration force and depth</td>
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</table>

2.4. Data and statistical analysis

2.4.1. Penetration depth
The width of the microneedle at the location where it penetrated the top surface of the skin was determined by analysing individual video frames (Fig. 2). From this width and the image of the microneedle shape (Fig. 1), the depth of the microneedle in the skin could be calculated. This was performed for multiple frames of each sample, each separated by approximately 0.1 mm displacement, using a custom written program in Matlab (R2013a, The MathWorks, Inc., Natick, MA, USA) and manually selecting the width of the microneedles in the images. The estimated depth of the microneedle was associated with corresponding values for penetration force and displacement.

To determine the influence of the skin donor on the penetration depth, a linear regression model was used with data of the measured penetration depths. Subsequently, the R-squared value was determined. To determine the influence of microneedle tip diameter, the mean penetration depth at every subsequent 150 ± 75 μm displacement was compared. A non-parametric Kruskal–Wallis test was performed followed by Dunn–Bonferroni post-hoc test to test for significant differences between microneedles per displacement range, with a significant value set at 5% (i.e. p < 0.05).

2.4.2. Evaluation of penetration depth measurements
To evaluate the use of video frames to determine the progression of penetration depth, two additional methods to determine the maximal penetration depth were used. Before each experiment, the microneedles were dipped in methylene blue. An image of the microneedle was recorded after each test. From this image, the maximum penetration depth of the microneedle was determined by measuring the distance from the tip until the location where no methylene blue was visible (hereafter called ‘depth according to the image’), similar to the method used by van der Maaden et al. (2014). These images also confirmed that none of the microneedles failed during the experiments. In addition, the penetration depth during retraction of the microneedle was measured using video frames (hereafter called ‘depth at retraction’). This was defined as the depth determined from the video frame corresponding to the moment the force during retraction became zero, because then the microneedle was still at its maximal depth within the skin.

2.4.3. Penetration force
As reported in previous studies, a sudden decrease in the force can be observed when the skin is penetrated (Davis et al., 2004; Khanna et al., 2010). These drops were also observed in our experiments. The initial drop in force was defined as the first decrease in force larger than 0.685 mN. This threshold value was determined by measuring the maximal noise of the force signal under the current conditions, and multiplying it by 5. For each sample, three parameters were determined, namely, the force at the initial drop, the displacement at the initial drop, and the total decrease in force during the initial drop.

To examine the differences in the three parameters with microneedle tip diameter, a one-way analysis of variances (ANOVA) with a Bonferroni post-hoc test was performed. Normality of the test data and equality of variance between the groups were verified with Shapiro–Wilk and Levene’s tests, respectively. The assumption of equality of variance was violated for one parameter (p < 0.05). All statistical analyses were performed using SPSS (IBM SPSS Statistics 22, IBM Corp., Armonk, NY, USA).

3. Results

3.1. Evaluation of penetration depth measurements
The method of using video recordings of the skin surface to determine the penetration depth was evaluated by comparing the depth according to the video at maximal displacement with the depth at retraction and depth according to the image. It was seen that the depth from the video at maximal displacement was slightly higher than both the depth at retraction and according to the image; the latter two values were similar. This indicates that the penetration depth measured with the video frames was overestimating the penetration depth. By comparing the depth of the video at maximal displacement (dmax) with the depth at retraction (dret) using a linear regression model (dmax = 0.75 × dret + 2.35 μm, R² = 0.78) it was determined that the depth was overestimated
3.2. Influence of skin donor

To determine the influence of skin donor on the penetration depth, experiments were performed on samples from 5 different skin donors using microneedles with a tip diameter of 5 \( \mu \text{m} \). Fig. 3 demonstrates that the penetration depth increased linearly with microneedle displacement (\( R^2 \) varied from 0.73 to 0.97 for the various skin donors). It is evident that the slopes of the linear regression models were generally within 12% of each other.

3.3. Influence of tip diameter

To determine the influence of microneedle tip diameter on the penetration process, the penetration experiments were performed with the four different tip diameter microneedles on at least 6 skin samples per diameter. In 2 measurements for both the 5 and the 37 \( \mu \text{m} \) diameter microneedles there was no drop in force larger than the threshold value of 0.685 mN and these 4 samples were excluded from further analysis.

3.3.1. Penetration depth

Although the mean penetration depth at the maximal displacement of 1.5 mm was approximately 250 \( \mu \text{m} \) for all tip diameters (Fig. 4), the progression to this depth varied considerably. For the smallest tip diameter of 5 \( \mu \text{m} \), the microneedle was smoothly inserted into the skin, which was characterised by a linear increase in depth with microneedle displacement. In contrast, for larger tip diameters the penetration depth was initially smaller than the 5 \( \mu \text{m} \) tip microneedle, as the skin was predominantly indented. This was followed by a sudden insertion into the skin, as reflected in the sudden increase in depth after approximately 0.75 mm displacement, particularly evident with the 37 \( \mu \text{m} \) tip microneedle (Fig. 4). In some samples, this sudden increase in depth was clearly visible on the video recordings as can be seen in Fig. 5. Subsequently, the penetration depth rapidly increased up to a similar magnitude to the 5 \( \mu \text{m} \) tip diameter. A significant difference between the microneedle groups was found for displacements from 0 to 0.9 mm (\( p<0.05 \)).

3.3.2. Penetration force

The measured force during penetration increased non-linearly with displacement up to forces of approximately 320 mN. The penetration depth at a certain force is shown in Fig. 6. Similar to the relationship between penetration depth and microneedle displacement (Fig. 4), the depth at a certain force was different for the four microneedle diameters. It was evident that the sharper microneedles penetrated the skin at lower forces than the more blunt microneedles.

The force and displacement at the initial drops and the total decrease in force during these drops were compared for the four microneedles (Fig. 7). The force at the initial drop linearly increased with tip diameter with means ranging from 20 to 167 mN. A statistically significant difference in the force at the initial drop between the largest and two smallest diameter tip microneedles was found (\( p<0.05 \)), together with a difference between the 24 \( \mu \text{m} \) and 5 \( \mu \text{m} \) tip microneedles. The total decrease in force during the initial drop also increased with tip diameter from 1.5 to 30.4 mN, with a significant difference between 5 and 37 \( \mu \text{m} \) tip diameters. There was no significant difference in displacement at the initial drop, which occurred at approximately 1.1 mm displacement. If the penetration depth within the displacement range where the initial drop in force was

*Fig. 4 – The effect of microneedle tip diameter on the penetration depth related to the microneedle displacement. The penetration depth was significantly different between microneedles at lower displacement. Significant differences between microneedles are indicated with * (\( p<0.05 \)). Number of data points is 2 to 21 per bar.*

*Fig. 3 – The penetration depth of a microneedle with a tip diameter of 5 \( \mu \text{m} \) at a range of displacements tested on different skin donors. Single measurements are shown for a total of 33 samples from 5 skin donors with an associated linear regression model (LRM) (\( R^2 \) is 0.79, 0.86, 0.73, 0.87, and 0.97, respectively). The slopes of the linear regression model for the different skin donors were 221, 141, 149, 149, and 133 \( \mu \text{m/mm} \), respectively. The diamonds, asterisks, squares, circles, and triangles represent single measurements of donor 1 to 5, respectively.*

by approximately 34%. Subsequent data shown was corrected by the value of the slope of the linear regression model.
measured (range mean ± standard deviation) was compared for the various tip diameters, it is worth noting that the penetration depth was approximately 160 μm for all groups. This suggests that the microneedles had already penetrated the upper layer of the skin and thus, at the time an initial drop in force was measured, penetration involved one of the deeper skin layers.

In more than 50% of tests with the three smallest tip diameters, more than one drop in force larger than the threshold value was recorded within a single test. By contrast, for the 37 μm tip diameter only one drop in force greater than the threshold value was evident within a single test.

4. Discussion

It is important that insertion of microneedles into the skin is controlled to ensure a reproducible depth of insertion when the technique will be adopted in the clinic. In the present study, the penetration depth and insertion force of single microneedles with various tip diameters, ranging from 5 to 37 μm, were determined. Recordings demonstrated a more smooth penetration of the skin by microneedles of smaller tip diameters. This was supported both by the measurements of the penetration depth, which increased more linearly for sharp microneedles than for blunter microneedles, and the sudden drop in penetration force, which was larger in magnitude for the blunter microneedles. Therefore, sharp microneedles with diameters less than 15 μm are essential to insert microneedles in a well-controlled manner to a desired depth.

In the present study, no difference in penetration depth was found between different skin donors. The measured penetration depths were slightly lower than reported in a previous study using micropipette pulled microneedles with a tip opening of 22 to 48 μm (Martanto et al., 2006a). These authors reported a penetration depth of 300 to 350 μm at a displacement of 1080 μm. This discrepancy can be due to the substrate employed, which was stainless steel in the previous study.

Fig. 5 – Video frames of the penetration of a 37 μm tip microneedle indicated a sudden increase in penetration depth. Top: from left to right three subsequent video frames at 15 frames per second. The dashed lines mark the edges of the microneedle. The width of the microneedle at the location where it penetrated the skin is indicated; bottom: the corresponding force curve. The dot shows the force corresponding to the video frame.

Fig. 6 – The penetration depth related to the measured force is shown in the figure per microneedle group.
Previous studies determined the penetration force of various microneedles by measuring a sudden decrease in force exerted on the microneedle (Davis et al., 2004; Khanna et al., 2010). The authors assumed that, at the moment a drop in the force was measured, the stratum corneum was ruptured. This interpretation is questioned by the present findings, since the initial drop in force corresponded to a measured penetration depth of approximately 160 μm for all tip diameters. This depth approximates to the depth of the transition from the papillary to the reticular dermis. The reticular dermis consists of a densely packed collagen fibre network and these fibres are likely to resist penetration by a microneedle. The present findings indicate that the penetration of the stratum corneum and viable epidermis was smooth in nature and therefore did not result in a measurable drop in the force even with a very sensitive load cell. Therefore, it is conceivable that previous studies also measured the penetration of a deeper skin layer. In many of the present experiments, more than one drop larger than the threshold value was measured within a single test. This indicated that the microneedles were gradually inserted into the skin, as previously suggested (Gittard et al., 2013). In some cases, drops in force were of smaller magnitude than the threshold values. These may represent penetration of the stratum corneum and/or noise in the force signal.

In the present study, the force at the initial drop linearly increased with tip diameter. This seems contrary to a previous study by Davis et al. (2004), who reported a linear relationship between the force at the initial drop and the tip area of the microneedles, with diameters ranging from 60 to 160 μm. However, pooling data from the two studies revealed an interesting relationship between force at initial drop and tip diameter, as illustrated in Fig. 8a. It is apparent that the force at the initial drop is linearly related to the radius (r) for sharp microneedles, while for tip diameters larger than approximately 80 μm, the force at the initial drop is proportional to r². This may be explained by the combination of forces the microneedle exerts on the skin (Fig. 8b and c). The microneedle stretches the skin surface, resulting in a high stress in the skin in a ring around the edges of the microneedle. The size of this ring is equivalent to the circumference of the microneedle tip, which is proportional to r. In
addition to stretching the skin, the force will be influenced by compression of the volume of skin underneath the microneedle, which is proportional to \( r^2 \). Results indicate that the force to stretch the surface dominates over compressing the skin at small tip diameters (Fig. 8b), while the inverse is the case at increasing tip radii (Fig. 8c).

The present study benefits from the use of a single microneedle to penetrate the skin. Previous studies measured the force of insertion of a microneedle array, and then divided this force by the number of microneedles in the array (Kochhar et al., 2013; Olatunji et al., 2013; O’Mahony, 2014; Roxhed et al., 2007). However, pilot studies performed with microneedle arrays (array of 4 x 4 microneedles of 300 \( \mu \)m) revealed that microneedles did not significantly penetrate the skin before the back plate of the array made contact with the skin, thus influencing the measured force. Therefore, when using an array, a measure of the force per microneedle will undoubtedly be influenced by the microneedle density within the array. By using a single microneedle without a back plate, we were able to accurately measure the force of a single microneedle. Although the insertion process may be different when microneedles are applied in an array, it is important to first understand this process for a single microneedle.

Specific aspects of the study design are worthy of discussion. First, the penetration depth was corrected to overcome the overestimation when measuring the microneedle width at its boundary with the skin. This overestimation was caused by the skin curling around the microneedle during the insertion process. Therefore, the visible boundary of the skin with the microneedle was not always equal to the point where the microneedle actually entered the skin. This effect was reported by Martanto et al. (2006b), who fixedate the skin with the microneedle in place. In the histological images, the deflection of the skin is visible. However, when the microneedle was retracted after penetration, the deflection of the skin recovered while the microneedle was still penetrating the skin, which made it possible to correct for the curling of the skin. Apart from this correction, it was estimated that the maximum error in determining the penetration depth using the video frames was approximately 30 \( \mu \)m. Second, Figs. 3 and 4 depict a larger spread in penetration depth above 1 mm displacement compared to depths under 1 mm displacement. This may be a result of the slight variation in skin thickness: depending on the sample thickness the PDMS substrate was indented to a larger extent. With a Young’s modulus of approximately 0.8 MPa, PDMS is stiffer than subcutaneous tissue, which has a Young’s modulus of 20–30 kPa (Gefen and Haberman, 2007). Therefore, the use of PDMS as a substrate instead of subcutaneous tissue, may have an influence on the absolute values found for the penetration depth in relationship to the microneedle displacement. However, this difference in substrate stiffness is not expected to unduly influence the major findings of the present study. Similarly, the relative humidity of the environment may affect the penetration process of the stratum corneum, since its stiffness is known to be dependent on the relative humidity (Geerligs et al., 2011b; Yuan and Verma, 2006). Nevertheless, the overall influence of the stratum corneum on the penetration process seemed to be limited. In addition, the present study was performed with human skin tissue, which has more physiological relevance to previous studies involving porcine or murine skin samples (Crichton et al., 2010; Donnelly et al., 2010b; Gittard et al., 2013).

To our knowledge, this is the first study that visualised the microneedle penetration depth and skin deflection during the insertion process of a single microneedle while measuring the force. By performing these experiments for microneedles with a range of tip diameters, a more complete insight into the penetration process was obtained. It can be concluded that microneedles with a smaller tip diameter penetrate the skin more smoothly than microneedles with a larger tip diameter. Therefore, if we want to controllably insert microneedles to a desired depth for the purpose of vaccine delivery, sharp microneedles (<15 \( \mu \)m) are necessary.

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