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**Title:** Microneedle-mediated vaccine delivery  
**Issue Date:** 2014-12-10
CHAPTER 3

Microneedle Pretreatment
1. Introduction

Microneedles are needle-like structures shorter than 1 mm that have been advocated as devices for enabling potentially pain-free intradermal delivery of biomacromolecules [1-5]. To permit a reproducible delivery of the drug, microneedles should be inserted into the skin in a controlled and reproducible manner [4, 6]. Obviously, one of the factors that influence the penetration ability of microneedles is the insertion process itself, e.g., microneedles can be inserted manually or by using insertion devices [3, 4, 6-9]. Recently, the penetration ability of low-density arrays (42 microneedles/cm²) with 750 µm long microneedles, applied manually or by using a snap-based applicator, was investigated [10]. However, no studies have been reported on the efficiency and reproducibility of the insertion of high-density microneedles into human skin. Therefore, the aim of this study was to investigate the effect of the type of application on inter and intra individual variability of microneedle insertion into human skin by microneedle users.

We show that participants using an impact-insertion applicator inserted high-density microneedles into ex vivo human skin with high efficiency and with a low inter and intra individual variation. However, when the same microneedle arrays were inserted by using a manual insertion device, the penetration efficiency was reduced by approximately 40% with a considerably lower reproducibility. Finally, the applicability of an impact-insertion applicator/microneedle arrays was confirmed in a vaccination study in mice.
2. Materials and methods

2.1 Microneedle application

In this study high-density arrays (576 microneedles on a 5x5 mm backplate, gifted by Bosch, Germany, Stuttgart) with a microneedle length of 200 µm were used (figure 1). The microneedles were applied onto ex vivo human abdominal skin that was obtained from hospitals within 24 hours after cosmetic surgery and dermatomed to a thickness of 600 µm [11]. To investigate factors that influence the penetration efficiency, microneedles were applied onto the skin for 10 seconds with different forces (3.43-22.1 N) by applying weight rods (350-2250 g) onto the insertion device for manual application. Also, the effect of application time (5-60 seconds) with a constant force (7.36 N) was investigated. Next, 15 participants (21-57 year; 10 male and 5 female) volunteered to apply a microneedle array three times onto ex vivo human skin, in a direction perpendicular to the skin surface, by using either a manual application device (figure 1D), or an impact-insertion applicator at a velocity of 3 m/s (figure 1E) [12]. The type of microneedle application was randomly performed.

2.2 Penetration ability of microneedles

To determine the penetration ability, 70 µL of an aqueous 0.4% trypan blue (Sigma Aldrich) solution was applied and left for one hour at the site of prior microneedle application. Subsequently, the skin surface was washed two times with water and once with 70% ethanol. Next, the stratum corneum (SC) was removed by tape-stripping (Scotch tape) until no SC residue was visually observed on the tape and the skin appeared shiny. Finally, the penetration efficiency (PE) was calculated as follows:

\[ PE = \left( \frac{\text{number of blue spots}}{576} \right) \times 100\% \]

2.3 Safranin staining

To visualize the SC removal efficiency, 10 µm thick cryosections of control and tape-stripped skin were made on a Leica cryostat (CM 3050S) and stained for 1 minute in a 1% (w/v) Safranin O solution (Sigma Aldrich). Next, the number of layers was visualized by swelling the SC in a 2% (w/v) KOH solution for 20 minutes (n=3).
2.4 Immunization of mice with ovalbumin

Eight-week old female BALB/c mice (Charles River) were immunized thrice with intervals of three weeks by the ‘poke and patch’ approach (100 µg ovalbumin /70 µL PBS (pH 7.4) for two hours, as previously described [13]), by using the impact-insertion applicator to apply either the high-density microneedle arrays or our first-generation LU-microneedles. The latter are made of 30 G needle tips, 300 µm long, and fixed in a backplate as a 4x4 microneedle array. They were previously shown to penetrate the skin when using the impact-insertion applicator [12]. A subcutaneous injection of 5 µg ovalbumin in 100 µL PBS was used as positive control. Ovalbumin-specific serum IgG responses were determined by a sandwich ELISA, as described previously [13]. Antibody titers were expressed as the log value of the serum dilution at the mid-point of a complete S-shaped absorbance-dilution curve. The study was carried out under the guidelines complied by the animal ethic committee of the Netherlands, and was approved by the “Dierexperimentencommisie Universiteit Leiden (UDEC)” under number 13065.

3. Results and discussion

3.1 Assessment of piercing efficiency after stratum corneum removal

The application of dyes at the site of microneedle application is commonly used to assess microneedle penetration in the skin [12, 14-16]. However, when observing the complete skin following microneedle application, the resulting spots are not always penetrations, but could be indentations of the SC (see supplemental information). Indeed, our study made clear that without SC removal the penetration efficiency is overestimated. When microneedle arrays were applied with a manual insertion device onto ex vivo human skin, blue spots were visible (figure 2A), suggesting successful skin piercing with an efficiency of 81% (figure 2C). However, after removal of the SC less blue spots were visible (figure 2B), showing that only 46% of the microneedles pierced the skin (figure 2A). The SC consists of 20 layers of corneocytes (figure 2D), and after tape-stripping most of the SC was removed (figure 2E), which allowed a reliable assessment of the piercing efficiency. Therefore, this approach was used in the study described below.

3.2 Penetration ability of high-density microneedle arrays

First, two factors, application force and time, that potentially influence the penetration efficiency of manual microneedle application were investigated (figure 3). Increasing the applied force (at a constant application time of 10 seconds) up to 7.36 N greatly improved the penetration ability (3A), which is in agreement with the literature [9]. Further increase of the force or prolonging the application time of the microneedles at a constant force (7.36 N) only minimally increased the penetration efficiency but improved the reproducibility (3B).

Next, to investigate whether persons without microneedle experience are able to successfully and reproducibly penetrate skin, 15 participants were instructed to apply...
microneedles onto ex vivo human skin by using a manual insertion device or an impact-insertion applicator (figure 3C and 3D). Using a manual application device resulted in low penetration efficiencies (56%) and relative high inter and intra individual variation, which was probably caused by variations in applied force and that the microneedle application conditions were below the optimum insertion conditions. However, when using the impact-insertion applicator, all participants pierced the skin with significantly lower inter and intra individual variation. These results show that using a microneedle applicator is essential for efficient and reproducible penetration of skin by a high-density microneedle array.

3.3 Vaccination study in mice

To demonstrate that the use of the impact-insertion applicator can lead to reproducible immune responses following microneedle-mediated immunization, we performed a vaccination study in mice, using ovalbumin as a model antigen. Figure 4 shows that microneedle-based vaccination leads to the induction of reproducible ovalbumin-specific IgG responses. Interestingly, using high-density microneedles resulted in up to ten-fold higher IgG responses as compared to our first-generation LU-microneedles. As both microneedle arrays result in reproducible piercing, the difference is probably caused by differences in microneedle density, number (576 versus 16 microneedles/array) and geometry [12]. Furthermore, a comparison between microneedle-based and subcutaneous administration revealed that vaccination by using high-density microneedles initially led to significantly lower IgG responses than subcutaneous vaccination, but the differences became negligible after the 2nd boost. This shows that microneedle-based vaccination can lead to comparable immune responses as
**Figure 3:** Penetration efficiency after application of a high-density microneedle array onto ex vivo human skin with different forces for 10 seconds (A) and at a constant force (7.36 N) with varying application times (B). The application of microneedles onto ex vivo human skin by non-experienced microneedle users (C and D). 15 participants pierced the skin with 200 µm long microneedles by a manual insertion device (open circles) or an impact-insertion applicator (closed circles). Each point represents the average penetration efficiency of three individual microneedle applications by one individual (C) and the relative standard deviation (RSD) of the penetration efficiency for each participant (D). Significance (**p<0.001**) was determined by an unpaired two-tailed T-test.

**Figure 4:** Ovalbumin-specific IgG responses upon microneedle-based (n=8) immunization by using an impact-insertion applicator with first-generation (LU-MN) or high-density (Bosch) microneedles, and subcutaneous (s.c.) injection (n=5). Each bar represents the mean ± SEM. Non-responders were given an arbitrary titer of 1 and significance (*p<0.05, ***p<0.001) was determined by a two-way ANOVA with a Bonferroni post test.
compared to conventional immunization. Similar or slightly lower responses of microneedle-mediated vaccination with ovalbumin, as compared to subcutaneous/intramuscular injection, has been observed before [17, 18]. In conclusion, these data show the potential of using high-density microneedles with an impact-insertion applicator for vaccination.

4. Conclusion

This study shows that using an impact-insertion applicator improves the efficiency and reproducibility of high-density microneedle insertion, enabling reliable self-application of microneedle arrays onto the skin. Moreover, it was demonstrated that the impact-insertion applicator can be used for microneedle-mediated antigen delivery, yielding robust antigen-specific IgG responses, which depend on microneedle density and/or geometry.

Acknowledgements

We thank Dr. Michael Stumber (Robert Bosch GmbH) for the supply of microneedles and Eleni Maria Varypataki and Stefan Romeijn for their help with the immunization study.

References

Supplemental Information

Cryosection of microneedle treated ex vivo human skin with a manual application device, showing three blue stains on the stratum corneum (1-3). In this example two out of three (2 and 3) blue stains are penetrations of the skin and one blue stain is an indentation of the stratum corneum.
CHAPTER 3.1: Microneedle pretreatment
Impact-insertion applicator improves reliability of skin penetration