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Application of in vivo Laser Scanning Microscopy to Visualise the Penetration of a Fluorescent Dye in Solution and in Liposomes into the Skin after Pre-Treatment with Microneedles

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1. Introduction

One of the most important functions of our skin is its barrier function. The skin keeps the water inside and protects us against environmental noxins such as light, pollutions or germs. Therefore, it is not easy to transmit substances into the skin even when it is desired. The uppermost layer of the skin, the horny layer (stratum corneum), acts as a physical barrier. Only small moderate lipophilic drugs can pass this barrier to achieve a therapeutic effect. For this reason the pharmaceutical industry has invested a high amount of effort in designing formulations or delivery methods to overcome the skin barrier and to enhance the penetration of active substances across the stratum corneum. One important novel area of research is the development of vaccines, which can be applied directly onto the skin without any pain sensation. However, the active part of the vaccine, the antigen, is a highly molecular weight substance, which makes it difficult to deliver by the dermal route using the more conventional methods.

One of the novel methods for the delivery of highly molecular weight substances through the skin barrier is the use of microneedles. Microneedles were introduced in the field of transdermal and dermal drug delivery more than a decade ago (Henry et al., 1998). Microneedles are shorter than hypodermal needles, but long enough to breach the stratum corneum barrier, the upper 20 μm of the skin. The application of 0.5 mm microneedles is described as painless (Kaushik et al., 2001). They have been developed as a replacement for the traditional needle and syringe and to facilitate the transport across the skin barrier without any pain sensation. When using solid microneedle arrays, small conduits are created in the skin, which are large enough to allow the penetration of high molecular

weight compounds across the stratum corneum (Prausnitz, 2004). Furthermore, compared to piercing with a conventional needle, there is only a minimal microbial infiltration through the conduits, and microorganisms do not reach the dermis (Donnelly et al., 2009). More recently, microneedles have also been used for vaccination (Ding et al., 2009, Kim et al., 2009, Koutsonanos et al., 2009, Matriano et al., 2002, Widera et al., 2006). Several studies have shown that the use of microneedles increases the efficiency of vaccination (Ding et al., 2009, Matriano et al., 2002, Van et al., 2009). In addition, microneedles can be used in combination with nanoparticles that may increase the transport of antigens or low molecular weight substances across the conduits (Bal et al., 2010a). However, in the development of these formulations, it is important to demonstrate the efficiency of transport by visualisation methods, preferably in vivo in humans.

The question is: How do we monitor the penetration? In this study, our aim was to estimate the size of the microneedle-induced conduits, their time behaviour and the diffusion of the active substances. In addition, it is of interest to monitor the efficiency of transport when the compound is encapsulated in liposomes.

There are several methods to study the skin penetration of substances, most of which are performed in vitro or invasively (Benfeldt, 1999, Gregoire et al., 2008, Rouse et al., 2007, Scheuplein, 1967, Schmook et al., 2001, Teichmann et al., 2005, Toll et al., 2004, Vogt et al., 2003, Weigmann et al., 2005). One non-invasive in vivo method is confocal laser scanning microscopy (CLSM) which can be used to visualise cell structures and to monitor the penetration of fluorescent dyes into the skin (Ardigo et al., 2010, Dietterle et al., 2008, Eichert et al., 2010, Lademann et al., 2003, Lange-Asschenfeldt et al., 2009, Martschick et al., 2007, Meyer et al., 2007). To show the ability of CSLM in vivo, a fluorescent dye (fluorescein) was injected into the epidermis using a syringe (Meyer et al., 2006). The dye is mostly distributed in the intercellular layers providing a strong contrast of the cellular structure in the CLSM images, as is shown in figure 1, in which three horizontal skin layers are shown. The cell structure of the corneocytes can be recognised (Fig 1a) and the reduction of cell diameter with increasing depth (Fig 1b). Furthermore, the papillary structure in the stratum basale can be observed (Fig 1c).

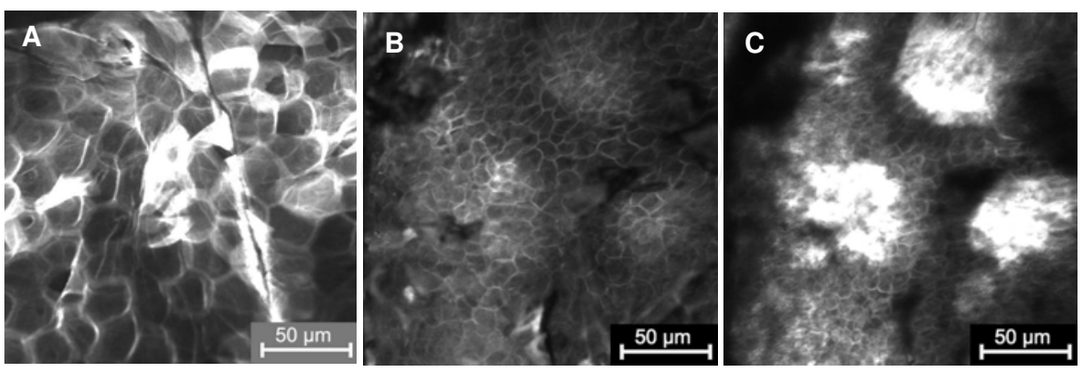


Fig. 1. CLSM images of A) stratum corneum at the surface, B) stratum spinosum at 70 µm, C) stratum basale with papillary structures at 110µm.

If the dye is applied on the surface and hair follicles or sweat glands are available, the dye will penetrate into the hair follicles and can be monitored (Meyer et al., 2007). Fig. 2 shows an example of a hair follicle and of a sweat gland at a depth of approx. 20 µm, again in in vivo settings.

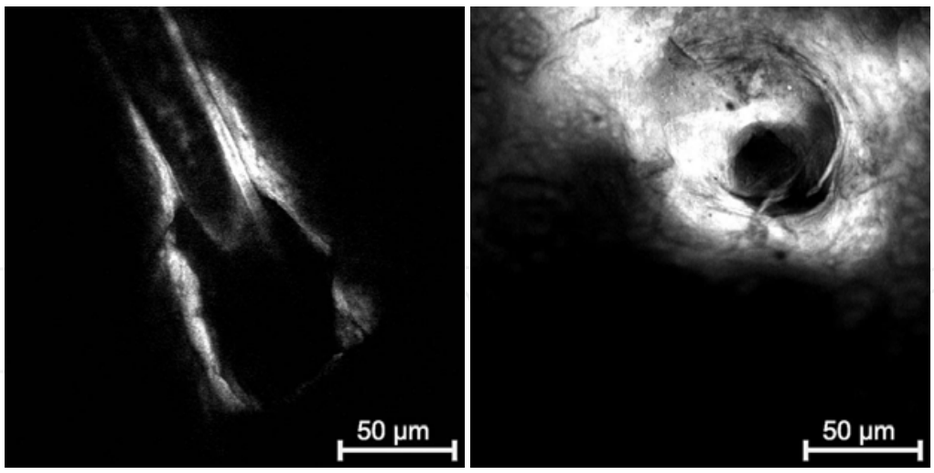


Fig. 2. CLSM images of a hair follicle (on the left) and a sweat gland (right).

These images illustrate that penetration into orifices can be monitored using CLSM. Therefore, it is highly probable that the conduits formed by microneedles can be monitored over time using CLSM.

The information obtained from CLSM studies of skin penetration can be used to optimise formulations for delivery of substances into the skin in general or into special parts of the skin, for example, transport through hair follicles or sebaceous glands (Grams et al., 2005, Lademann et al., 2009, van den Bergh et al., 1999). Often, these investigations were performed in vitro on porcine ear skin or excised human skin. In vitro, kinetic measurements can be performed, which can provide very useful information. However, measurements in the in vivo settings are more relevant, but suffer from the fact that only a limited number of dyes can be used.

CLSM can be applied both in vitro and in vivo. For example, Meyer et al. imaged Malassezia yeasts on living human cutaneous tissue using sodium fluorescein to label both the skin surface and the micro-flora structures with this method (Meyer et al., 2005). As well as visualising the penetration of substances into the skin, the effect of encapsulation of a dye into particles on the penetration pathways can be studied (Ossadnik et al., 2006), and CLSM can be used to study morphological changes of the skin (Dietterle et al., 2008, Eichert et al., 2010, Meyer et al., 2007).

Recently, we showed that by using CLSM it is possible to visualise the conduits made by solid microneedle treatment in human volunteers (Bal et al., 2010b) and to compare different microneedle systems (Bal et al., 2010c). One of these microneedle arrays was selected to compare the penetration of a fluorescent dye applied in solution with that applied in liposomes.

The microneedle arrays that were used in the present study have already been proven to be painless (Bal et al., 2008) and were shown to be efficient in enhancing the immune response when applying Diphtheria Toxoid as an antigen (Ding et al., 2009). In the present study, the model drug fluorescein was used.

2. Methods and materials

2.1 Volunteers

Six healthy volunteers (5 females and 1 male), aged between 20 and 58 years (mean 33 years) with no pre-existing skin conditions participated in the study with the fluorescent dye. The

study had been approved by the Ethics Committee of the University Hospital Charité (Berlin, Germany) in accordance with the Rules of Helsinki. As a pilot study, the penetration of the fluorescent dye encapsulated in liposomes was visualised in one volunteer.

2.2 Microneedle system

Stainless steel microneedles were prepared by electrical discharge machining (300ED). The microneedles have a square base of 250 x 250 µm and a length of 300 µm and are also positioned in a 4x4 pattern with a pitch of 1.25 mm. The shape of the tip is defined by a diagonal plane, which runs from the top of one side of the square pillar to the opposed bottom, in this way forming an angle of approximately 40° relative to the bottom surface (figure 3).

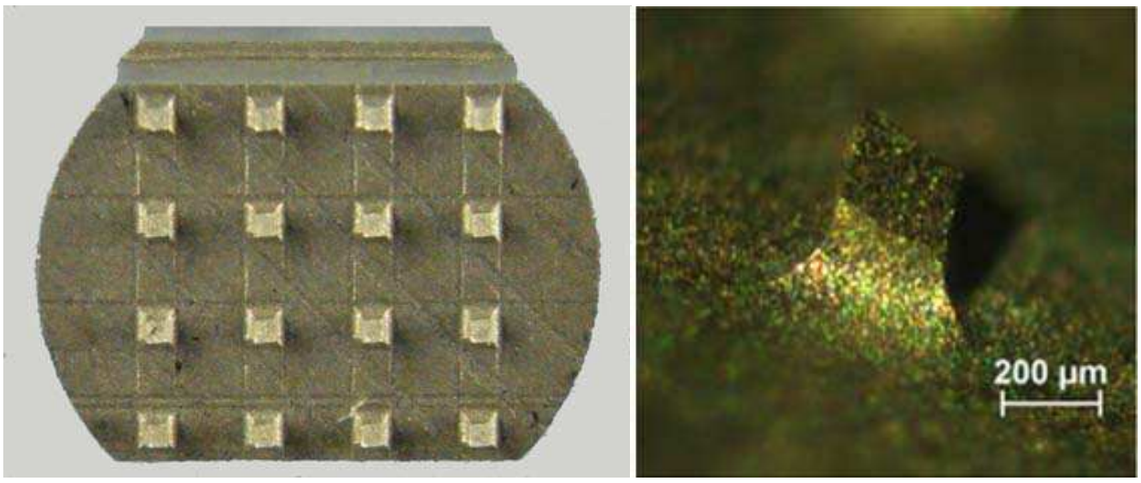


Fig. 3. Array (left) and the applied microneedle more detailed (right).

To apply the microneedles in a controlled manner, an electrical applicator was used, as described previously (Verbaan et al., 2007). With this applicator the microneedles are applied onto the skin at a speed of 3 m/s. To visualise the conduits, 0.2% solution of sodium fluorescein was used (Alcon Pharma GmbH, Freiburg, Germany).

2.3 Preparation of liposomes

Fluorescently labelled liposomes were prepared from soybean phosphatidylcholine (Lipoid GmbH, Ludwigshafen, Germany) using the film hydration method followed by extrusion. A thin lipid film was formed at the bottom of a circular flask using a rotary evaporator. This film was hydrated in a 10 mM phosphate buffer pH 7.4 (7.7 mM Na_2HPO_4 and 2.3 mM NaH_2PO_4) containing 2 mg/ml fluorescein. The dispersion was shaken in the presence of glass beads at 200 RPM for 2 h at room temperature and subsequently extruded 4 times (LIPEX™ extruder, Northern Lipids Inc., Canada) through a carbonate filter with a pore size of 400 nm and 4 times through a filter with a pore size of 200 nm (Nucleopore Millipore, Amsterdam, The Netherlands). Unincorporated label was removed by filtration using a Vivaspin 2 centrifugal concentrator (PES membrane, MWCO 30 kDa, Sartorius Stedim, Nieuwegein, The Netherlands). The final lipid-to-fluorescein ratio was 1:2 (w/w). In this manner, liposomes of 143 ± 9 nm in size were obtained.

2.4 Set up of in vivo confocal laser scanning spectrometer

For the investigations, a CLSM Stratum® (Optiscan Ltd., Melbourne, Australia) was used to visualise the epidermal tissue in vivo. Stratum® has a hand-held optical scanner which is connected by optical fibres with the photo-detector of the basic system. The optical window of the hand-held device is placed directly on the skin. Fig. 4 shows a schematic diagram of the optical principle of Stratum®.

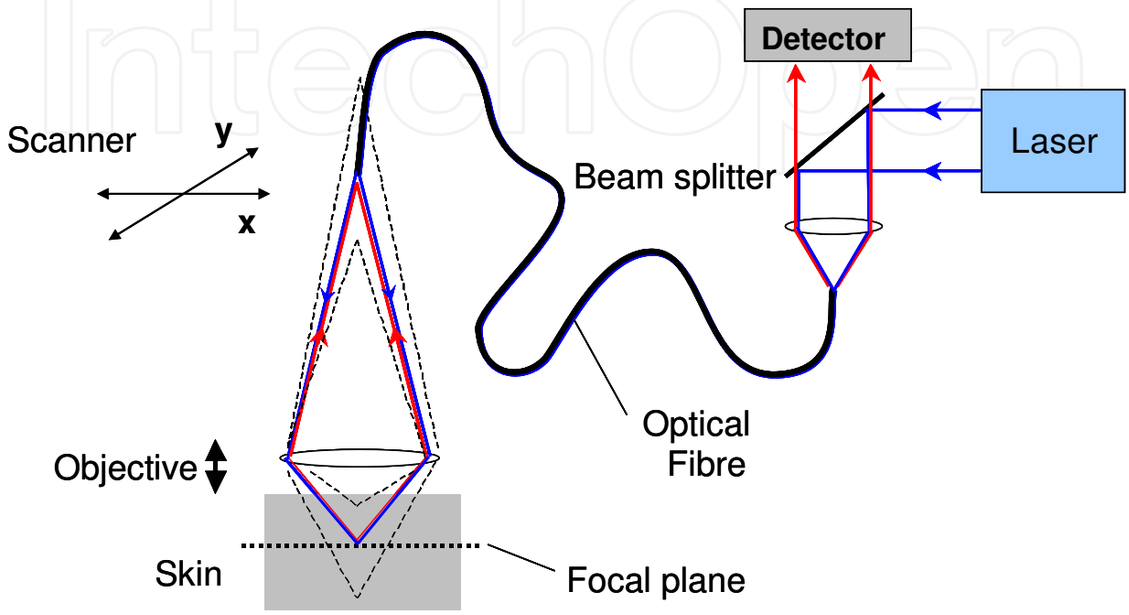


Fig. 4. Schematic diagram of the fibre based in vivo CLSM. Only the fluorescent light of the focal plane can enter the entrance of the fibre.

The illumination source of the Stratum® system is a blue 488 nm single-line argon ion laser. The scanned field of view is 235 by 235 µm² and the magnification is approximately 1000x. The obtained images are parallel to the skin surface resulting in horizontal or en face sections. The confocal imaging is restricted to the epidermis and superficial papillary dermis (Suihko et al., 2005). The imaging depth is varied by adjusting the focal plane manually with the focus tuning unit on the hand-held device.

2.5 Experimental procedure

The microneedles and the formulations were applied onto the ventral forearms of the volunteers. The skin was disinfected before the application of the formulations and the microneedle. The experiments were performed in triplicate on each volunteer. The fluorescein solution (50 µl / 0.8 cm²) was applied after the skin had been treated with the microneedles. The dye was applied for 1 minute and removed with a tissue paper. Afterwards, the conduit was visualised with CLSM; 5, 10 and 15 min after application images were taken at different depths. At least 5 images were taken to monitor the dye at the surface, the lateral and vertical distribution and the maximal penetration depth. Between the measurements the laser was set out of focus to avoid bleaching. As a control, a drop of fluorescein was applied to untreated skin. This control experiment showed that at the concentration used in this study no bleaching of the fluorescein occurred.

Investigations with fluorescein encapsulated liposomes were performed on the forearm of 1 volunteer. One drop of fluorescein encapsulated liposomes as described above was applied after piercing with microneedles. The liposomes were removed using soft tissues after 1 minute of application. The CLSM evaluation was performed in the same way as in the in vivo study with fluorescein. The measurements were performed in triplicate.

2.6 Data analysis

The images were analysed with respect to fluorescence pixel intensity and area using Image J (National Institutes of Health, USA). The pixel intensity was categorised into three different classes: the class with the highest pixel intensity was set between 230 and 255 AU and the signal was referred to as "high intensity fluorescence" (HIF); the class with pixel intensity between 230 and 14 AU is referred to as "low intensity fluorescence" (LIF) and the class with pixel intensity values below 14 AU is regarded as background. The autofluorescence of the skin was always below 14 AU.

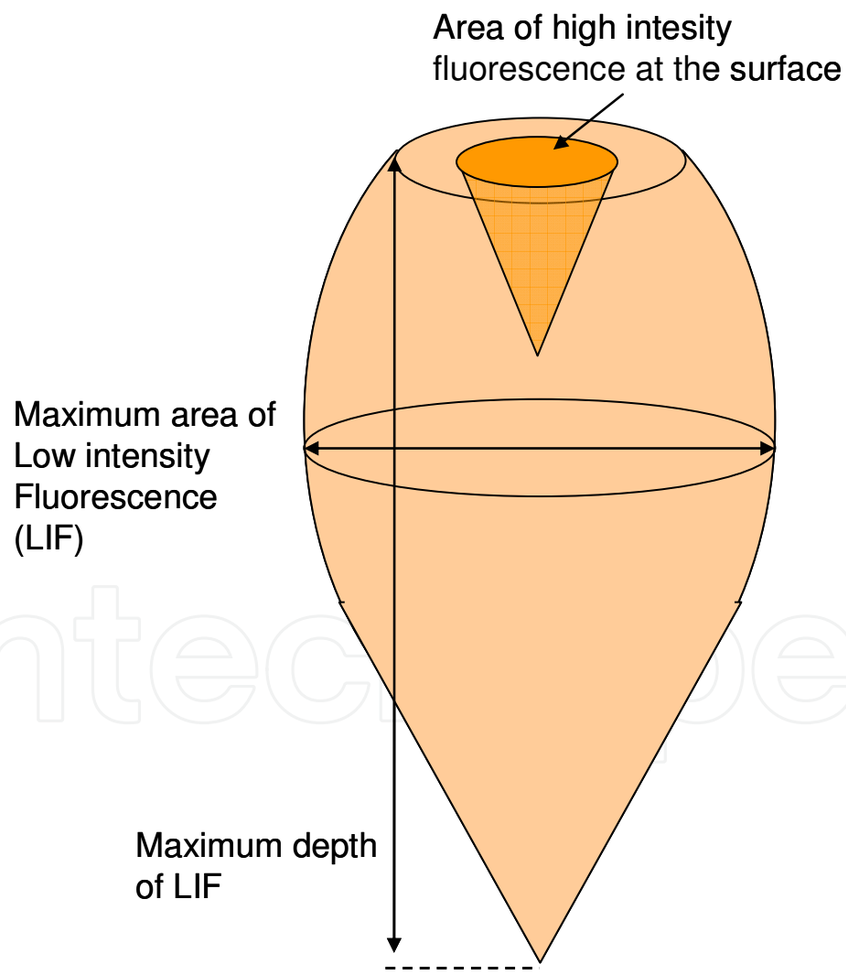


Fig. 5. Schematic diagram of the conduit formed by microneedles and the analysed parameters.

These thresholds were selected based on analysing 31 random images of different depths taken from two volunteers. The thresholds were selected in such a way that at least 90% of the test pixels were inside the described classes. The fluorescent signal from other skin structures such as furrows or hair follicles was removed manually. The area of either HIF or LIF was calculated by the number of pixels in the specified intensity areas. The following parameters were analysed: the area of HIF at the surface, the maximum area of LIF in the skin and the maximum depth where LIF can be detected. The parameters are further explained in figure 5.

3. Results

In Fig. 6, representative CLSM images of the fluorescent dye in a conduit formed by microneedles at different time points and at different depths are shown.
The HIF is clearly visible at the surface 5 min after removal of the formulation, thus the stratum corneum can easily be identified. The fluorescent dye preferably diffuses through the lipid regions surrounding the corneocytes, outlining the cells. The HIF at the surface decreases with time and after 15 min it has vanished. At the deeper skin layers a more diffuse LIF signal is present. From these images it appears that the largest area of HIF was present at the skin surface at all time points. However, this was not always the case. In

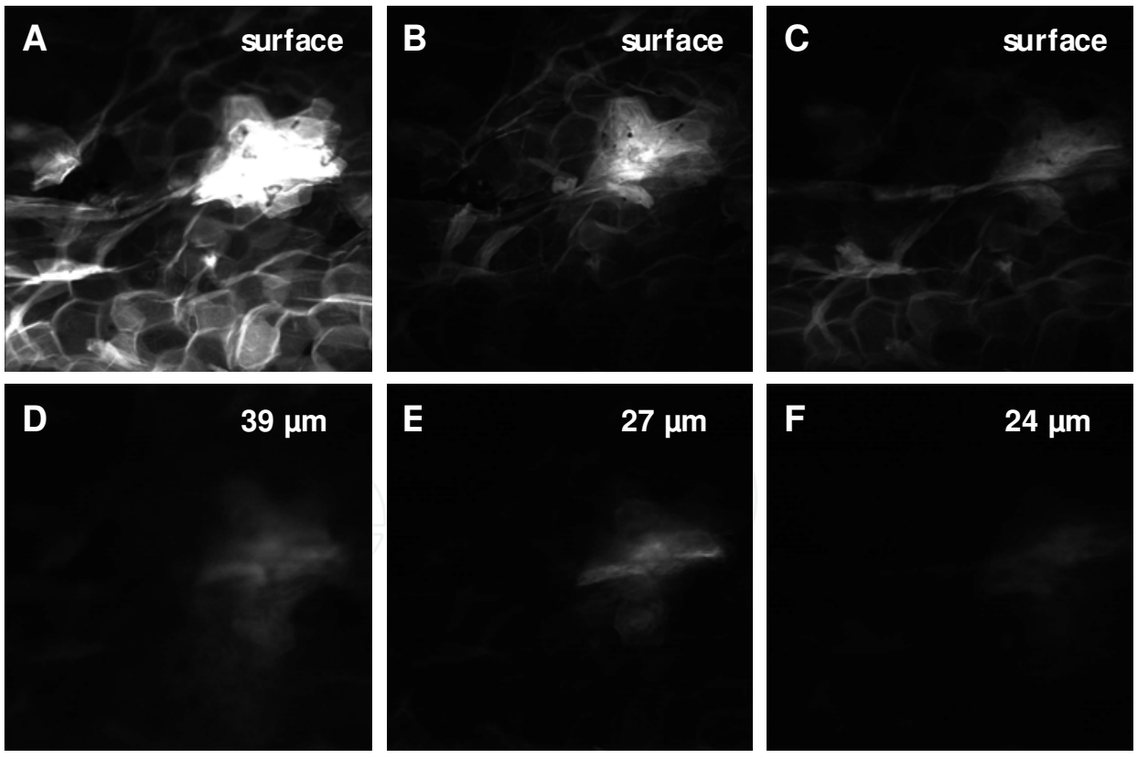


Fig. 6. Representative CLSM images of conduit closing over time in vivo after piercing and application of fluorescein in solution. Conduit at the surface at A) 5 min, B) 10 min and C) 15 min. At the indicated depths, the maximal diffusion area at D) 5 min, E) 10 min and F) 15 min was found. Field of view: 235 by 235 µm^2.

approximately 35% of the images, the largest area of HIF was observed below the surface, but within the upper 20 µm of the skin, in the stratum corneum.
In Fig. 7, representative CLSM images of conduits over time after piercing and application of fluorescein encapsulated liposomes are shown. The HIF is clearly visible at all time points at the surface and additionally up to a depth of 94 µm after 5 min. The LIF was observed in larger areas compared to the images obtained when fluorescein in solution was applied.
The changes in the area of HIF versus time are shown in Fig 8. for both formulations.

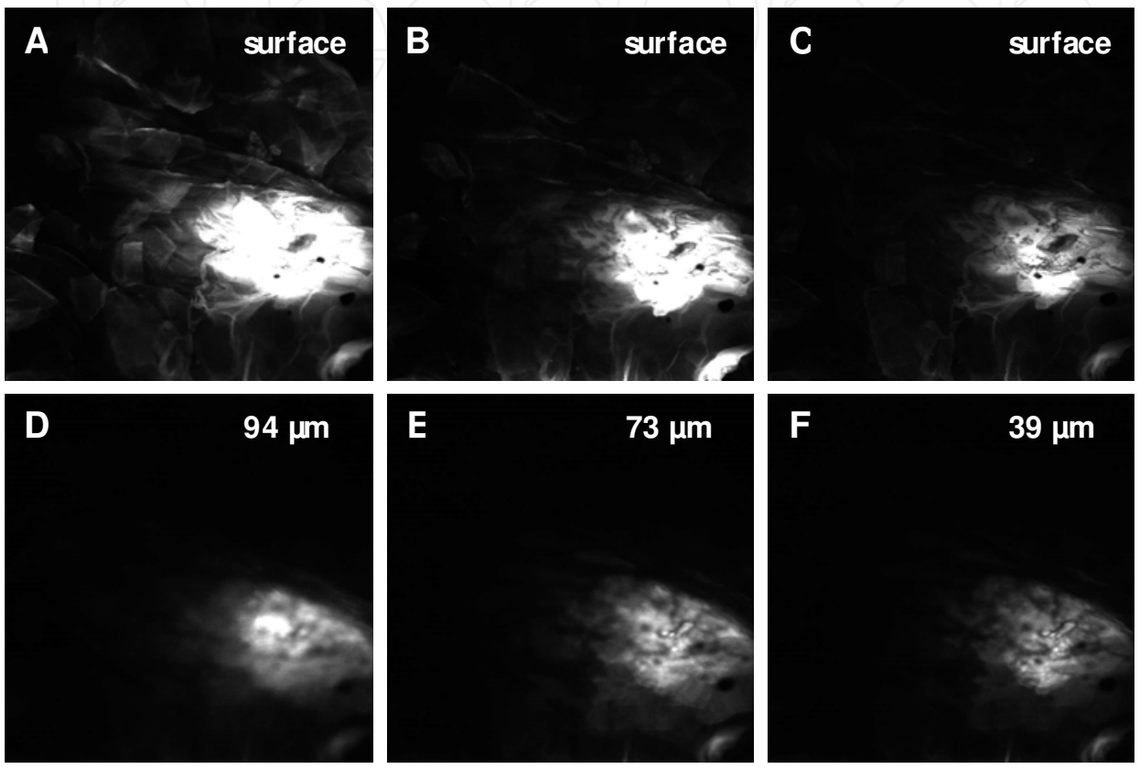


Fig. 7. Representative CLSM images of conduit closing over time after piercing and application of the fluorescein encapsulated liposomes. Conduit at the surface at A) 5 min, B) 10 min and C) 15 min. At the indicated depths, the maximal diffusion area at D) 5 min, E) 10 min and F) 15 min was found. Field of view: 235 by 235 µm².

At all 3 time points the area of HIF at the surface was elevated after liposome application compared to a fluorescein solution. Nevertheless, both showed a similar decay in HIF in time.
In Fig. 9, the maximum area of LIF is shown for both formulations as function of time.
In contrast to fluorescein encapsulated liposomes, the maximal diffusion area for the fluorescein solution decreased substantially between 5 and 10 min. In the time interval between 10 and 15 min, the opposite trend was observed; the maximum area of LIF for fluorescein encapsulated liposomes decreased substantially more than that of the fluorescein solution. Importantly, the maximum areas of LIF of fluorescein encapsulated liposomes were constantly higher compared to a fluorescein solution.
The time dependence of the maximum depth of LIF is shown in Fig. 10 for both formulations.

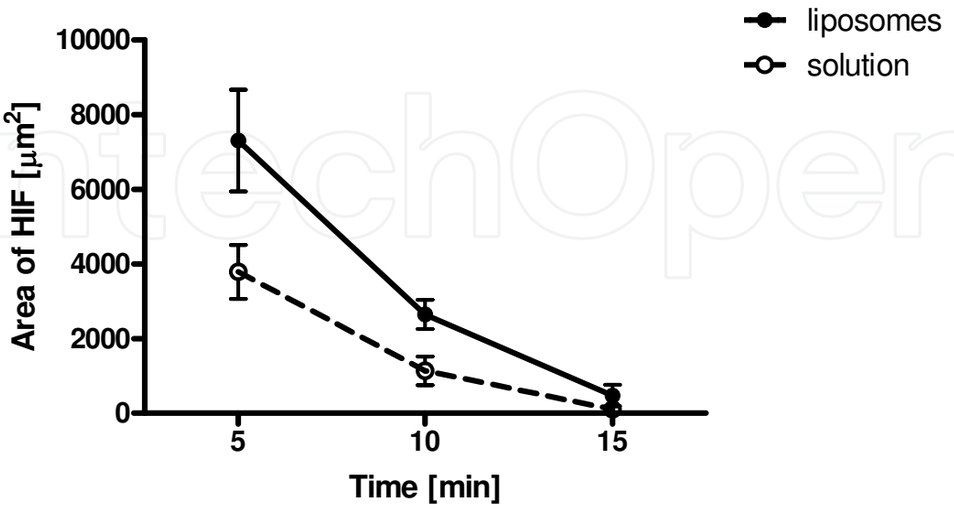


Fig. 8. Time dependency of the HIF area at the surface (µm²) for applying fluorescein encapsulated liposomes and fluorescein solution after microneedle piercing. Data are presented as mean value ± SE, n=3 for particles and n=18 for solution.

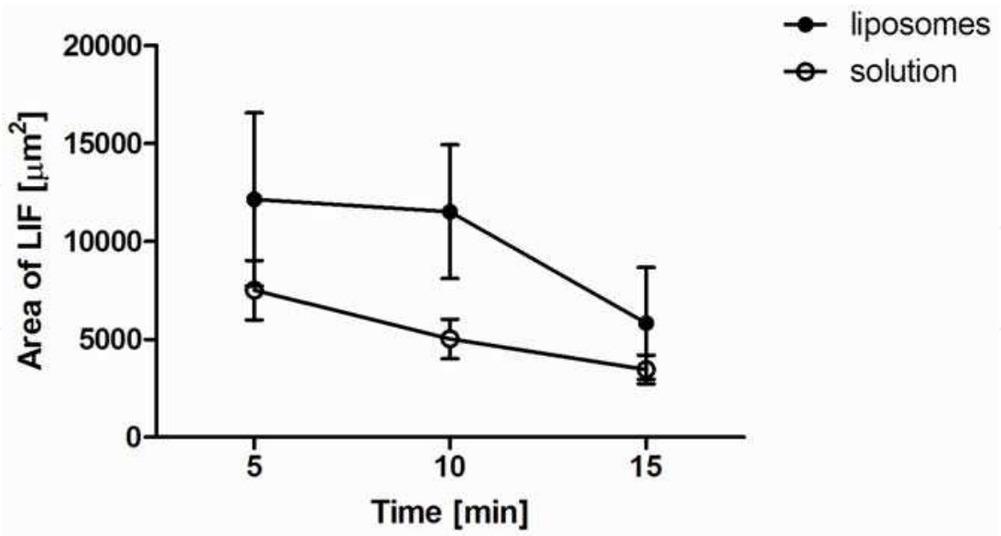


Fig. 9. Time dependence of LIF area for applying fluorescein encapsulated liposomes and fluorescein solution after piercing. Data are presented as mean value ± SE, n=3 for particles and n=18 for solution.

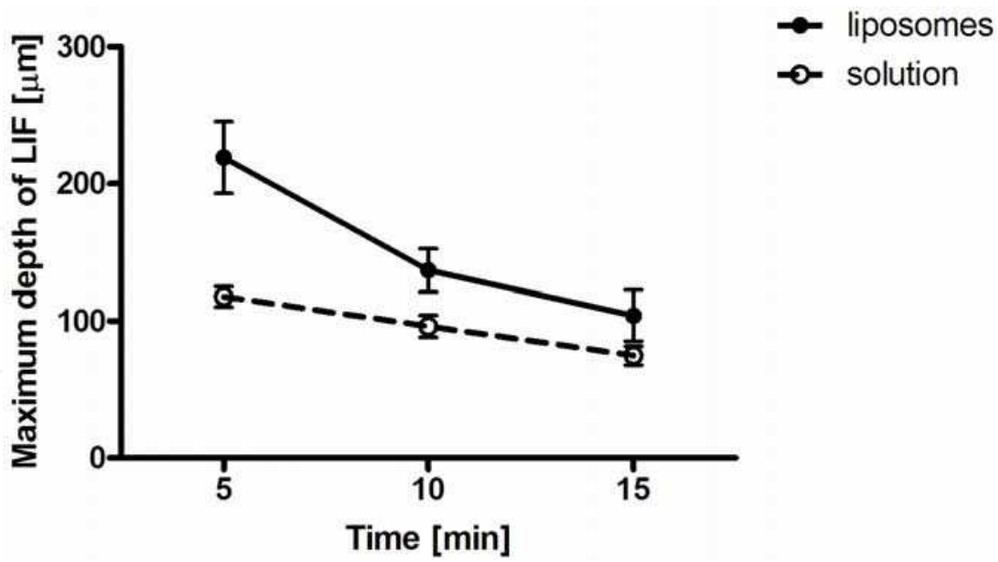


Fig. 10. Time dependence of the mean maximum depth of LIF (µm) for applying fluorescein encapsulated liposomes and fluorescein solution after piercing. Data are presented as mean value ± SE, n=3 for particles and n=18 for solution.

The maximum depths of the LIF of the solution were less compared to the liposome formulation. Moreover, the changes in time of the maximum LIF area were less distinct, in particular the change between 5 and 10 min.
These studies emphasise the potential of microneedles for cutaneous vaccine delivery and of using CLMS to monitor fluorescent species in microneedle conduits in vivo over time.

4. Discussion

The ultimate purpose of microneedles is to deliver active substances more rapidly and in sufficient amounts across the skin barrier to achieve a therapeutic effect. The results clearly demonstrated that the CLSM is able to monitor a fluorescent penetration applied either in solution or encapsulated in liposomes.
The present pilot in vivo study with fluorescein encapsulated liposomes was carried out to evaluate the penetration of fluorescein applied in liposomes in comparison to an aqueous fluorescein solution. A statistical analysis could not be performed, because the liposome data were obtained from only one volunteer, in triplicate. The preliminary results clearly showed that the diffusion area of fluorescein applied in liposomes was increased compared to fluorescein applied in a solution. In particular at 5 min the maximum depth of LIF, and the HIF area were increased when applied in liposomes. This may be due to an increased accumulation of the liposomes in the conduits compared to fluorescein only. This is consistent with the findings of Verbaan et al., who observed a considerably lower passive transport efficiency across human skin after microneedle array treatment for a large complex of FITC coupled Dextran (MW 72 kDa) compared to the smaller compounds Cascade Blue (MW 538) and Dextran – Cascade Blue (MW 10 kDa). This was observed for all examined needle lengths: 550, 700 and 900 µm. Nevertheless, all 3 compounds were significantly increased compared to the transport rate across the skin, when the skin was not pre-treated by microneedles (Verbaan et al., 2007). Most probably, during the transport

along the conduits, the dye is already partially released from the liposomes. However, because the time points were only 5, 10 and 15 min after application, the dye in the conduits may, at least partially, still be encapsulated in the liposomes. We cannot exclude that the difference in penetration of the fluorescent label is also due to a difference in activity of fluorescein in the two formulations. Fluorescein was applied at equal concentration and not at equal thermodynamic activity.

In contrast to the maximum observed area of LIF and the maximum depth of LIF, the time dependence of the decrease in area of HIF was remarkably comparable for both studies. Nevertheless, the preliminary results of the liposomes should be validated by further investigations.

5. Conclusion

In these studies, we have shown that CLSM is an excellent method to monitor the fluorescent distribution in time of a free fluorescence label, and the label incorporated in liposomes along conduits in human skin, in vivo. The pilot study shows an increased area of HIF at the skin surface and an increased maximum area of LIF within the tissue, as well as an increased depth of the fluorescent signal when liposomes were used.

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Ever since the invention of laser by Schawlow and Townes in 1958, various innovative ideas of laser-based applications emerge very year. At the same time, scientists and engineers keep on improving laser's power density, size, and cost which patch up the gap between theories and implementations. More importantly, our everyday life is changed and influenced by lasers even though we may not be fully aware of its existence. For example, it is there in cross-continent phone calls, price tag scanning in supermarkets, pointers in the classrooms, printers in the offices, accurate metal cutting in machine shops, etc. In this volume, we focus the recent developments related to laser scanning, a very powerful technique used in features detection and measurement. We invited researchers who do fundamental works in laser scanning theories or apply the principles of laser scanning to tackle problems encountered in medicine, geodesic survey, biology and archaeology. Twenty-eight chapters contributed by authors around the world to constitute this comprehensive book.

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