In vitro indentation to determine the mechanical properties of epidermis

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ABSTRACT

The lack of understanding of the mechanical behavior of the human skin layers makes the development of drug delivery using microneedles or microjets a challenging task. In particular, the key mechanical properties of the epidermis composed of stratum corneum and viable epidermis should be better understood.

Micro-indentation experiments were applied, using a spherical tip with a large diameter to the sample thickness ratio. The Young’s moduli were derived via an analytical and a numerical method. The tests showed that the analytical method was not appropriate to assess the Young’s moduli. That is why a numerical model was used to obtain the correct stiffness. When loaded perpendicularly, the stiffness of both the epidermis and stratum corneum vary between 1 and 2 MPa. No significant differences in stiffness between the stratum corneum and viable epidermis were observed.

1. Introduction

The outer skin layer possesses characteristics that make it a favorable site for pain-free drug delivery with minimal damage. Indeed, it presents a rich population of immunologically sensitive cells as well as the lack of blood vessels and sensory nerve endings. The development of drug delivery using microneedles or microjets is a challenging task because of deficient understanding of the mechanical behavior of the human skin layers. In particular, the key mechanical properties of the outer skin layer, i.e., the epidermis composed of stratum corneum and viable epidermis, should be better understood.

The structure and function of this layer are well-known (Elias and Feingold, 2005). The outer layer, the stratum corneum, is an effective physical barrier of dead cells in a “brick-and-mortar” structure: the anucleate corneocytes form “bricks” and the intercellular lipid membranes and corneosomes are considered to represent the “mortar”. The viable epidermis mainly consists of keratinocytes migrating towards the stratum corneum, continuously changing in composition, shape and function. The junction with the underlying dermis is strengthened by its undulating pattern, such that large cones of epidermal tissue penetrate the dermis (see Fig. 1). Furthermore, epidermal properties are influenced by environmental factors such as temperature, humidity and UV radiation.

In order to deliver drugs transdermally, the microneedle or microjet should penetrate the stratum corneum to deliver the drug 100–150 μm below the skin surface, e.g. in the viable epidermis or papillary dermis. A full understanding of the delivery path requires also the understanding of this indentation phase and, therefore, the knowledge of the mechanical behavior of the epidermis.

Recently, Kendall et al. (2007) were the first to report on the mechanical properties of the (viable) epidermis during penetration, using modified standard tips on murine skin. They observed a decrease in storage modulus when the 2 μm probe penetrates through the stratum corneum, which is in accordance with studies on stratum corneum only (Plewig and Marples, 1970). The authors explained this by an increase in moisture content with depth. In the viable epidermis, the storage modulus remained nearly constant. By contrast, penetration of the 5 μm probe showed a negligible decrease in storage modulus throughout the stratum corneum and a gradual increase in the viable epidermis, although the values of the shear moduli were less than that for the corresponding 2 μm probe.

A variety of in vivo and in vitro indentation techniques were developed to measure the stratum corneum. In the eighties, Hendley et al. (1982) developed an indentation device to measure force variations in vivo due to age, sex and body site. A needle with a tip radius of 11 μm at the tip was held perpendicular to the surface and moved rapidly into the skin. They claimed that the speed of the indentation ensured that the test was predominantly confined to the properties of the stratum corneum (Graves and Edwards, 2002). Measured forces were typically in the order of 3.0 N. Recently, a limited number of nano-indentation studies have been performed on isolated stratum corneum (Pailler-Mattei et al., 2007; Pailler-Mattei and Zahouani, 2004; Plewig and Marples, 1970). The tips used varied between 1 and 10 μm, while corneocytes have a diameter ranging from 26 to 45 μm (Holbrook and Odland, 1974). As a consequence,
very local properties were determined in these experiments. Furthermore, in some of the studies, the three-sided Berkovich tip, that has a sharp three-sided point, is used. This tip easily induces damage on the sample’s surface, which interferes with the load-displacements results. Three of the nano-indentation studies were based on continuous stiffness measurements (CSM) protocols (Kendall et al., 2007; Pailler-Mattei et al., 2007). The drawback of CSM is that the results are influenced by the selected amplitude and frequency for viscoelastic materials. Combining the nano-indentation studies on stratum corneum reveals measured Young’s moduli varying from 10 MPa (Yuan and Verma, 2006) for wet porcine samples up to 1 GPa for dried human samples (Pailler-Mattei et al., 2007). This broad range is likely caused by the differences in testing apparatus and protocols, differences between species and body sites, and the heterogeneity of the material. A reliable method to determine the mechanical properties of the stratum corneum on the tissue level only is therefore also required.

The aim of the present study is to present such an indentation method and to use it to determine the Young’s modulus of the epidermis, i.e., the stratum corneum and viable epidermis. The typical complex geometry, a variable thickness between 30 and 150 μm, and the porosity of the epidermis places high demands on this mechanical characterization. Therefore, isolated epidermis and isolated stratum corneum were tested using equipment that is known for its accuracy and reliability. The device is originally designed for solid materials of which well-defined samples can be obtained and therefore the testing protocol needs to be adapted to epidermal samples. To validate that the testing protocol holds for thin materials with a low stiffness, tests have been performed with silicone rubber. Moreover, indentation experiments require a model for the interpretation of the measured results. Next to the analytical model used, which assumes a homogeneous linear elastic material behavior upon unloading, we also adopted a numerical model that allows for taking into account geometrical details and different material properties for different layers.

2. Methods

2.1. Sample preparation

Indentation tests have been carried out on ex vivo abdominal skin of Caucasian women from a similar age 43 ± 4 years old undergoing abdominoplasty surgery. All patients gave informed consent for use of their skin for research purposes under a protocol approved by the ethics committee of the Catharina Hospital, Eindhoven, The Netherlands. Abdominal skin with striae markers, cellulite, damage due to UV exposure or excessively hairy skin is excluded from the study.

Immediately after excision, the skin was brought into the laboratory and processed within 4 h. Epidermal sheets were obtained using a dermatome (D42, Humeca) in which the prescribed thickness was refined for this purpose by the supplier. The dermatomed slices of 100 μm thickness were cut in pieces of approximately 1 cm². Depending on various factors such as skin surface roughness, tissue hydration and the amount of cones and ridges (see Fig. 2), samples may consist of epidermis and/or some papillar dermis (see Fig. 1b and c).

To obtain stratum corneum samples, dermatomed skin slices of 200 μm were immersed in a solution of 0.1% trypsin (Hyclone, SV30037.01) in an incubator at 37 °C for 2–3 h. Thereafter, the sheets were rinsed in PBS and also cut into pieces of approximately 1 cm². All samples were stored at −80 °C until further use.

In order to validate the experimental procedure is valid for thin samples, a highly elastic silicone rubber (Köriform 42A, Alpina Siliconee, Germany) was measured using various sample thicknesses. The silicone rubber was poured under vacuum into various thicknesses: 0.05, 0.12 and 2.0 mm. Thereafter, samples of about 1 cm² were cut out.

Fig. 1. An aldehyde-fuchsin staining is used to visualize the morphology of the various skin layers: (a) full-thickness skin including the stratum corneum (SC), viable epidermis (VE), papillar dermis (PD) and reticular dermis (RD), (b) dermatomed skin with a set thickness of 100 μm consisting of the epidermal layer only and (c) dermatomed skin of 100 μm consisting of epidermis and some fragments of papillar dermis.

Fig. 2. The top center of the triangles, highlighted by the large red points, formed by the glyphics was chosen as indentation location on the skin samples. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
2.2. Experimental procedure

The skin sample should be placed on a substrate such that in-plane tissue movement cannot occur. The large number of pores in the epidermis precluded the use of any fixation method. It appeared, however, that the adhesive, sticky nature of the skin sample was sufficient and no further fixation was required. Immediately after thawing at room temperature, samples were spread out on an aluminum disk with the outer skin surface facing up. Possible air or liquid below the tissue was gently squeezed out. The samples were allowed to acclimatize for 20 min before the first indentation commenced.

On each skin sample, nine indentation locations were manually selected with use of the built-in microscope of the Nanoindenter XP (MTS Systems, USA). Each location is at least 500 μm away from the others to avoid any influence between measurements.

The centers of triangles formed by the glyphics, the primary and secondary lines, were chosen as indentation locations to optimize the contact between the indenter and the tissue (Fig. 2).

All experiments are performed using a sapphire sphere with a radius of 500 μm. The load and displacement resolutions are 1 nN and 0.01 nm, respectively. The maximum load depends on the depth limit of indentation, which was set to a value, which did not exceed 10% of the sample thickness (Oliver and Pharr, 1992). Preliminary testing demonstrated that this indicates a maximum load of 0.2 mN for stratum corneum and 1 mN for epidermis. The loading/unloading rate was 0.01 mN/s. The maximum load was held for a period of 30 s. For both epidermis and stratum corneum, the protocol was repeated on three samples for each subject. Test series were completed within 2 h. The temperature and humidity are kept constant at 22 °C and 28% RH, respectively.

The skin samples, particularly the stratum corneum samples, are extremely thin (under 20 μm). Measuring such thin samples might be at the resolution limit of the apparatus. Therefore, to evaluate the usefulness of the protocol for thin materials, a well-defined homogeneous soft material, silicone rubber, with different thicknesses (50–2000 μm) was tested with the indentation protocol similar to that for the epidermis. The samples were placed on the substrate without fixation. Indentation locations were indentified automatically, using a 3 × 3 grid with a distance of 500 μm between the various locations.

2.3. Determination of the Young's modulus

2.3.1. Analytical approach

In order to derive a first estimate of the Young's modulus, the experimental data of the skin and silicone rubber samples are analyzed by the method proposed by Oliver and Pharr (1992), which assumes a fully elastic recovery upon unloading and was originally developed for metals. From the initial unloading slope of the load–displacement (P, h) curve, the reduced modulus \( E_r \) is obtained according to:

\[
E_r = \frac{\sqrt{\pi} \Delta P}{2 A \Delta h}
\]

where \( A \) is the contact surface. In practice, the measured tip displacement is never equal to the contact depth because, at the vicinity of the tip, the surface can either sink-in or pile-up (see Fig. 3). In that case, \( A \) is replaced by the projected area \( A_p \), which can be calculated for small deformations according to:

\[
A_p = \pi a^2 = \pi (2R + h_1) a
\]

Subsequently, the Young's modulus is calculated following:

\[
E = \frac{1 - \nu^2}{E_r} = \frac{1 - \nu^2}{h_0^2 + h_0^2}
\]

where \( E \) and \( \nu \) are the Young's modulus and the Poisson's ratio, respectively, for the specimen and \( E_i \) and \( \nu_i \) for the indenter and \( E \) the measured reduced modulus. The epidermis, stratum corneum and silicone rubber are all assumed to approximate incompressible materials, using a Poisson's ratio of 0.495.

2.3.2. Numerical model

To check if the Young's moduli obtained with the analytical method give reasonable results, a finite element calculation using MSC.Marc (MSC.Software Corporation, Santa Ana, USA) was performed. An axisymmetric mesh was used to fit the experiments using a NeoHookean model with different material parameters when modeling stratum corneum and viable epidermis, assuming incompressible material behavior. The mesh consisted of 4329 linear quad4 elements, using full integration. The size of the mesh was chosen such that the edges do not influence the stress distribution and contact between the indenter and the sample was assumed to be frictionless.

For the silicone rubbers, the Young's modulus, \( E_{SB} \), was estimated by fitting the average load–displacement curve of the 50 μm thick samples. The value for \( E_{SB} \) was then used to calculate the unloading curves of the 120 and 2000 μm thick samples. These unloading curves are compared with the experimental data.

Since the deformations were small, linear elastic behavior was assumed for the skin samples too. First, the Young's modulus for the stratum corneum, \( E_{SC} \), was derived by fitting the average load–displacement curve of the stratum corneum samples of 20 μm. This modulus was used to describe the experimental data of the epidermis, such that the modulus for the viable epidermis \( E_{VE} \), could be derived.

The thickness of the stratum corneum was varied from 10 to 20 μm, the thickness of the viable epidermis was kept constant at 80 μm. In order to assess the sensitivity of this fitting approach, the effect of increasing \( E_{SB} \) or decreasing \( E_{SB} \) by a factor 2 on the maximum indentation depth was studied.

3. Results

The load–displacement curves obtained from the silicone rubber samples are shown in Fig. 4. The results were highly reproducible for each thickness. The maximum indentation depth slightly decreases with decreasing sample thickness. Consequently, the slope of the initial unloading curve decreases, which is reflected in the average values for the Young's moduli namely, 3.67 ± 0.20, 2.22 ± 0.10 and 1.69 ± 0.04 MPa for a sample thickness of 50, 120 and 2000 μm, respectively. It is evident that the assumption of a linearly elastic half space from Oliver and Pharr (1992) is not valid for these thin samples. Using the FE model,
the Young’s modulus was estimated to be 2.16 MPa. When using this value to describe the unloading curves for the 120 and 2000 μm thick sample, it is shown that the unloading curves and maximum indentation depth for all thicknesses are in good agreement with the experimental data (Fig. 5).

An example of the results from one subject is shown in Fig. 6. Data that displayed significant measurement errors or deviated from the general response were ignored. The measurements that are neglected in the analysis primarily concern issues where initial contact could not be found. This is explained due to the extreme low stiffness of the material. Since the nano-indenter uses a change in stiffness value as a criterion for initial contact, and we are working on the limit of detection of the machine, in some cases initial contact could not be defined. We also think that sometimes the contact between the indenter and the tissue was not optimal, likely because of the rough surface or unwanted horizontal movement of the tissue. This resulted into unrealistic load–depth curves, so it was very clear that these measurements were wrong and should be excluded. In general, 2 or 3 tests out of a series of 9 measurements were excluded from subsequent calculations. Fig. 7 clearly shows that the average curves overlap for different subjects. Estimates for the Young’s moduli were derived via the analytical approach and found to be 2.6 ± 0.6 and 1.1 ± 0.2 MPa for the stratum corneum and epidermis, respectively.

For a 20 μm thick stratum corneum and 80 μm thick viable epidermis, $E_{SC}$ is identical for $E_{VE}$ with a value of 0.6 MPa. Decreasing the thickness of the stratum corneum to 10 μm hardly affects $E_{SC}$. Also increase in the stiffness of the stratum corneum did not have an effect. However, a reduction in the stiffness of the much thicker viable epidermis causes an increase in indentation depth, from approximately 8 to 12 μm.

4. Discussion

For the tests in the current paper we decided to use a nano-indentation technique, which is fairly new, but readily available for metals. However, to apply the tests to stratum corneum we had to solve some major issues, especially concerning tissue geometry (roughness, very thin layer) and sample preparation to see whether or not the theoretical models and protocols used for metals could also be applied to skin samples.

The major problem in performing indentation experiments on skin is probably the skin’s surface roughness. In order to average out surface defects, we used a large spherical indenter (Ø $= 500 \mu m$) such that the contact area was much larger than the diameter of an individual cell, therefore homogenizing the applied deformation. During preliminary tests, that were performed close to the glyphs, it was observed that the poor contact definition in those areas resulted in an unacceptably high variability per subject. When
positioning the indenter at the highest point between a triangle formed by the glyphics, (see Fig. 2) establishing a well-defined contact between indenter and tissue was proved possible. In addition, the use of a spherical tip prevents stress concentrations and contact between indenter and tissue was proved possible. In addition, the use of a spherical tip prevents stress concentrations and avoids damaging the sample (Ebenstein and Pruitt, 2006). Using this protocol, highly reproducible data could be obtained. For all subjects the variance was negligibly small.

In order to obtain reproducible data from in vitro experiments, a correct sample preparation is essential. In this study, the epidermal samples were isolated using a dermatome. This method does not allow for separation of the epidermis at the basal membrane only, however, the advantage is that the bottom side of the sample with this obtained geometry is in full contact with the substrate. As only small deformations were applied, the results are not influenced by the possible fragments of papillary dermis in the sample. Current tests were performed with epidermis that was thawed and immediately used in a dry environment. As increase in moisture content in the epidermis decreases the stiffness, it becomes more difficult to define the initial contact surface at higher humidities. The current set-up only allows measurements in dry conditions and it is not trivial to redesign the set-up to work at different, controlled humidities, although there are no principle limitations. Because moisture may have a large influence on the results this should at this moment be considered a major drawback.

Initially, for the analysis of the experiments, the analytical method of Oliver and Pharr was used. It provides an easy method to assess the order of magnitude of the Young's modulus from the experimental data. However, this theory holds for homogeneous materials responding fully elastically upon unloading only and is developed for thick layers. Therefore these results are only used as initial estimates for a more sophisticated analysis. In the case of non-elastic behavior piling-up and sinking-in can be observed as described in the methods section. Due to piling-up of the tissue, the projected contact area is larger than that used in the calculations (see Fig. 3). In the present study, the deviation is small, because the large spherical indenter reduces these boundary effects.

The thin layer and in the future possibly a more complex description of the constitutive behavior require the introduction of a numerical model. In the current work, the numerical model was primarily used to check the effect of boundary conditions on the result and to enable a first estimation of the stiffness of the viable epidermis with a two layer model (not possible with the Oliver and Parr approximation). Thus, the numerical analysis should be considered a pilot study. In the near future it is our objective to use the numerical model in an inverse parameter optimization procedure to estimate both the stratum corneum as well as viable epidermal Young's moduli in a single iterative loop. It is foreseen that we can also extend the model to include viscoelastic behavior.

The results show that the stiffness of the viable epidermis is comparable to that of the stratum corneum. For both epidermal layers, the stiffness of the two layers is approximately 1 MPa, which shows that the viable epidermis considerably contributes to the mechanical response of skin at this length scale. In comparison with literature using indentation tests, current values for stratum corneum are on the low side of the published range (Wu et al., 2005; Yuan and Verma, 2006; Pailler-Mattei et al., 2007). This can be explained by the fact that the local properties studied in literature were mainly determined by the stiffness of the individual corneocytes, while our studies focused on the tissue level. In comparison with values for full-thickness skin stiffness from in vivo indentation tests, our values are two orders of magnitude higher (Jachowicz et al., 2007; Pailler-Mattei et al., 2007; Boyer et al., 2009).

The results demonstrate that the stiffness of the viable epidermis is comparable to that of the stratum corneum for perpendicular loading direction at a length scale relevant for clinical and cosmetic treatments. The applied load in this study covers the physiologically relevant range. For clinical applications such as transdermal drug delivery, the large deformations and the ultimate goal, the failure behavior of the epidermal layer need to be understood. The methods presented in this study are considered to be a suitable tool that can be extended for these purposes.

Conflict of interest statement

None declared.

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