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STRATUM CORNEUM BIOMECHANICS

INTRODUCTION

As the outermost layer of the skin, the stratum corneum participates in the functional properties of the skin (1). For some functions, i.e. photoprotection (2, 3) or barrier protection (4), it is well accepted that the stratum corneum plays the primordial role. Concerning the mechanical properties of the skin, the influence of the mechanical properties of the stratum corneum is also recognized (5,6), but its exact level of importance is still in debates as it doesn't exist clear results in the literature.

The stratum corneum could be considered as a composite material mainly made of corneocytes, intercellular lipids, corneodesmosomes, and other intercellular proteoglycans. Such a complex material should be characterized in a multi-scale approach in order to relate mechanical properties of the main constituents to macroscopic properties of the skin such as softness, firmness, elasticity, and tonicity.

This chapter is divided in three parts as it explores stratum corneum biomechanics at three different scales: the cellular level with descriptions of the corneocyte mechanical properties, the tissue level through mechanical properties obtained on in vitro stratum corneum layer, and finally at the organ level through the description of the contribution of the outermost layer, as a part of a multi-layer organ. Mechanical properties at the different scales will be described on normal stratum corneum, and under variable hydration states, in order to improve our global understanding on water interactions with.

Finally, for the three parts, we'll present a short state-of-the-art review, as well as new results obtained recently in our labs.

BIOMECHANICAL BEHAVIOUR OF THE STRATUM CORNEUM CELL CONSTITUANTS IN VITRO AND THE EFFECT OF HYDRATION.

INTRODUCTION

Corneocytes form the elemental brick in stratum corneum. From the deeper layers of the skin, the keratinocytes undergo strong structural and biochemical changes that end up with the comparatively harder corneocytes (refs). The membrane and cell's interior are dramatically changed, forming the well known penta or hexagonal shape cells of typically $\sim 40\mu\text{m}$ diameter and $\sim 200\text{ nm}$ thickness. The stiffness of the corneocytes originates from the internal developed network of keratin, a sulfur containing fibrous protein.

It is important to know the intrinsic mechanical properties of these cells to fully understand the stratum corneum mechanics. One approach is to look into extracting the individual cells allowing not to be influenced by the underlying stratum corneum layers.

There are two main sampling methods available to isolate individual corneocytes from the stratum corneum tissue,: the detergent scrub and the tape stripping techniques. The details of these techniques can be found elsewhere(1)

CURRENT RESARCH

Several studies have look into the deep ultrastructure of these highly keratinized cells often using optical and electron microscopy methods. Very detailed descriptions are found on the morphology and its variability depending on age, area of the body, external agents like water, etc.

Many observations come from two dimensional observations. In the particular case of Electron Microscopy (EM), the preparation process is relatively invasive, and the nature of the cells may be modified. For example, until recently, biologists needed to make use of the latter vacuum techniques to image surface samples, thereby preventing in-vivo analysis, since,

in general, biological materials cannot withstand prolonged vacuum. Remarkably improved techniques for specimen preparation allows for routine observation of skin tissue. Using an osmium based method Mihara(8) for example described in great detail the corneocytes arrangement among them (presence of interdigitations of the membrane for example) at the surface of skin tissue of limb origin. The undulating, folded surface membrane was evident from the images obtained. Transmission Electron Microscopy (TEM) gives more details of the transversal sections, emphasizing that the uneven and cracked cytoplasmic surface. Barton et al(3), using EM and Differential Phase interference light microscopy observed a similar structure on isolated corneocytes.

A description of more quantitative approach is to measure the dimensions of corneocytes are described by a review of Corcuff and Leveque(7). In essence, parameters like the projected area are easily obtained by optical microscopy but others like thickness and morphology are not straightforward. In effect, the corneocytes relatively small thickness is close to the limit of classical light microscopy. Scanning Electron Microscopy (SEM) provides, as we have seen, good qualitative descriptions but does not provide direct access to the exact vertical thickness dimension. Confocal Microscopy (CM) can allow for a better representation of the tridimensional structure although the resolution is still limited. Finally interferometric methods can have a good vertical resolution (of the order of 10 nm).(10) Atomic Force Microscopy (AFM) extends the field of information given by classical methods like EM or CM, allowing for example, reaching a very high resolution of biological structures in ambient conditions. Several AFM applications are reported in the literature.(11-12). In particular Kashibuchi et al. have measured in detail morphological parameters as volume, average thickness and real surface area of corneocytes coming from different anatomical location, age and pathological condition. The study emphasizes the importance of considering the three dimensional characteristics of the corneocytes.

While there are detailed cellular morphological descriptions in the literature, there are few studies that deal with the actual mechanical properties of these cells. Many experiments are “indirect” observations that are based in the morphological observation of the corneocytes and their change due to hydration for example. In effect, Keratin affinity for water is at the origin of the mechanical changes that are observed under hydratization, where water acts as a plastifier increasing the cell volume. For example Richter has looked recently at this effect using AFM. Main height, area and volume are measured in air and water. The swelling observed ($50 \pm 10 \%$) is mainly a thickness change with no significant lateral modification.

A more direct approach has been done by L veque et al.(13). The authors recorded the force necessary to elongate isolated corneocytes immersed in water using a micromanipulation technique. A fine glass probe was used to stretch individual corneocytes or to separate two adjacent ones, while the deformation was monitored with a video recorder. The calculated Young’s elastic modulus $E \sim 4.5 \cdot 10^8 \text{ Pa}$. is considered to be underestimated by the authors due to technical difficulties. Moreover these elastic properties are attributed to the cell membranes due to its high cystine content. In the case of cell adhesion, in some cases outermost corneocytes did not easily separate suggesting that desmosomes remnants are still present.

AFM NANOMECHANICAL APPROACH

We describe here some new advanced methods using AFM that are being used for a more detail determination of the mechanical properties of corneocytes.

Being AFM relatively new for skin applications, we will give a basic description: The instrument is based on the interaction of a sharp point (tip) attached to the end of a cantilever with the surface of interest. The interaction is measured by the cantilever bending that is itself monitored by the displacement of a laser beam reflected on the back of the cantilever to a XY

detector. The lateral movement of the tip or the surface is accomplished using a piezo-electric element which provides scanning capabilities. By monitoring the displacement of the tip, topographical images are easily obtained. In the dynamic modes (also known as *tapping* modes) the tip vibrates close to the surface, just enough to be sensitive to the van-der Waals potential. These modes have extended the applications to the visualization of living cells, tissues or even the visualization of liquids and biomolecules.

Our group has been pioneering in using an internal developed repositioning technique to follow in situ the morphological changes of corneocytes under the effect of external agents like water or humidity. Fig. GL1 shows an example of a corneocyte isolated and deposited on a silicon wafer surface. The already discussed complex surface membrane topography is observed with nanometer precision.

pre-peer-reviewed version

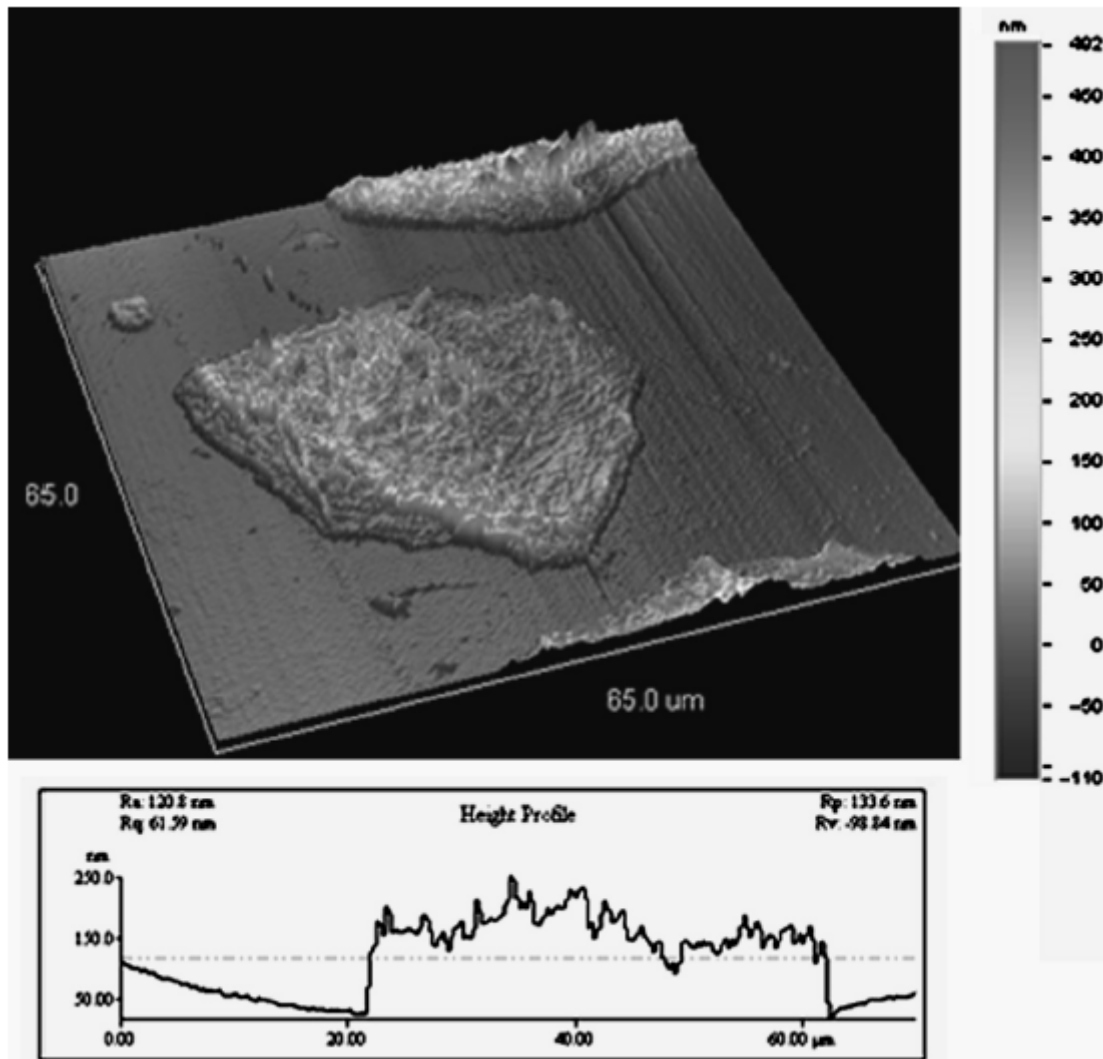


Fig. GL1. Example of an AFM image of an isolated stratum corneum cell (corneocyte) on a glass surface. This image contains all the topographic information (lateral and vertical) of the cell, as is illustrated by the height profile underneath.

As we have discussed, imaging with AFM is the result of the tip scanning over the surface. But the same interaction can be further analyzed as it also contains information about the local mechanical properties once the tip is in contact with the substrate. Although there are still many uncertainties about the physics concerning contact mechanics of AFM tips on substrates, many comparative studies have turned out to be very satisfactory, opening for a whole new spectrum of research.(14-17).

In essence, the scanner brings the sample and the tip together increasing then the load while monitoring the force induced on the cantilever that holds the tip and measuring the corresponding deformation (nano-indentation). The system records both the approaching and retracting curve, as force applied versus distance traveled by the piezo. The retracting or unloading curve is usually the one that holds information regarding the strength of the interaction; a minimum in the retracting curve (pull-off force) is associated to the adhesion. Depending on the load applied on the surface (loading force) the tip may indent the substrate. The degree of penetration can give information about the Young modulus of the material. Figure GL2 shows the typical parameters taken into account in these curves.

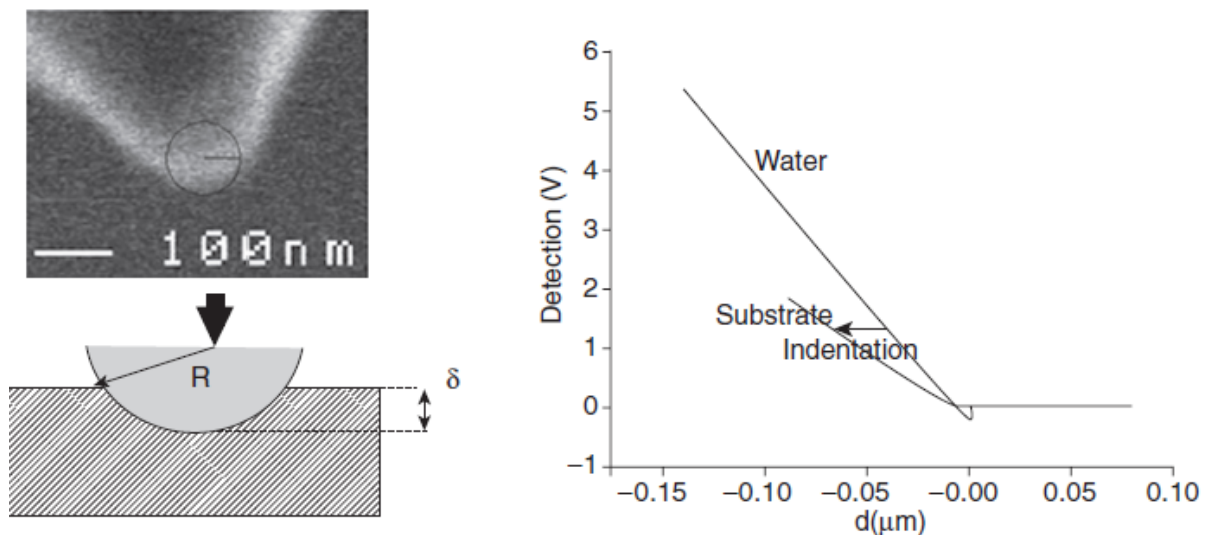


Fig. GL2. Scanning electron microscopy (SEM) micrograph (top) that shows the aspect of the apex of a commonly used Si₃N₄ AFM tip (A sphere is usually a good approximation to apply basic Contact Mechanics theories). Example of two indentation curves (bottom) performed on the surface of interest (green trace) and a reference hard material (red).

In our experiments we used Silicon wafers as references for infinitely hard surfaces. Surface mechanical indentations are then performed on these reference substrates previous to the corneocyte's experiments that were done in ambient conditions (~45% relative humidity).

The measured deflection curves are analyzed using Hertz's and Sneddon's elasticity models to extract the elastic modulus of the material. The Hertzian model for a spherical tip and a flat surface is given by:(18)

$$F = \frac{3}{4} E \sqrt{R} \delta^{3/2} \quad ,$$

where E is the elastic modulus, δ the indentation depth and R the radius of the sphere. This model, works well at low indentation depths. At higher loads and soft materials, the Sneddon model or even more sophisticated ones like the JKR (Johnson, Kendal, Roberts) {Israelachvili, 1980 209 /id} might be more appropriated. Our studies have proven that the Hertz model works reasonably well for the surface elastic characterization of isolated corneocytes, as long as the indentation depths are small enough (~ 10 nm) relatively to the corneocyte thickness.

We present the application of this method on a corneocyte cell in air. (ref. Baghdadli et al. 2007) Several surface indentations were performed at different positions in an squared array (local zones of $\sim 5\mu\text{m}^2$ in $\sim 10 \times 10$ arrays, see Fig. GL3). Apart from helping to average the values obtained, another advantage of using this approach is that we can extract information on specific zones of the surface of the corneocyte by superposing the obtained topographic image

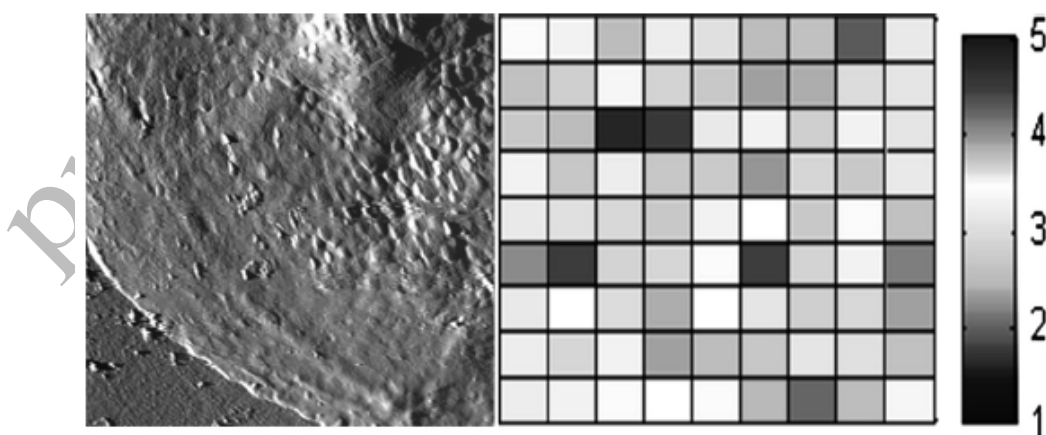


Fig. GL3. Example of a topographic AFM image ($30 \times 30 \mu\text{m}$) of a corneocyte (top) with its corresponding Elastic Young's modulus ($\times 10^9$ Pa) array map (below) obtained following the procedure described in the text.

At our low loads we did not observe any indents at the surface. This observation reconfirms that our experimental setting were consistent with being in the elastic regime of the corneocyte membrane deformation. The force curves obtained were then analyzed following the procedure that has been described above. The curve fits were satisfying and the elastic Young modulus extracted. The values that we obtain are in the order of $\sim 4.0 \cdot 10^9$ Pa and as expected bigger than those obtained in water by Leveque et al. {Leveque, 1988 2271 /id}. Further experiments are still needed to understand the effect of water using this direct method. Finally, this technique can also be applied at the surface of the stratum corneum with the advantage is that the surface mechanical elasticity can be monitored while applying a hydrating cosmetic like glycerol. In Fig. GL4

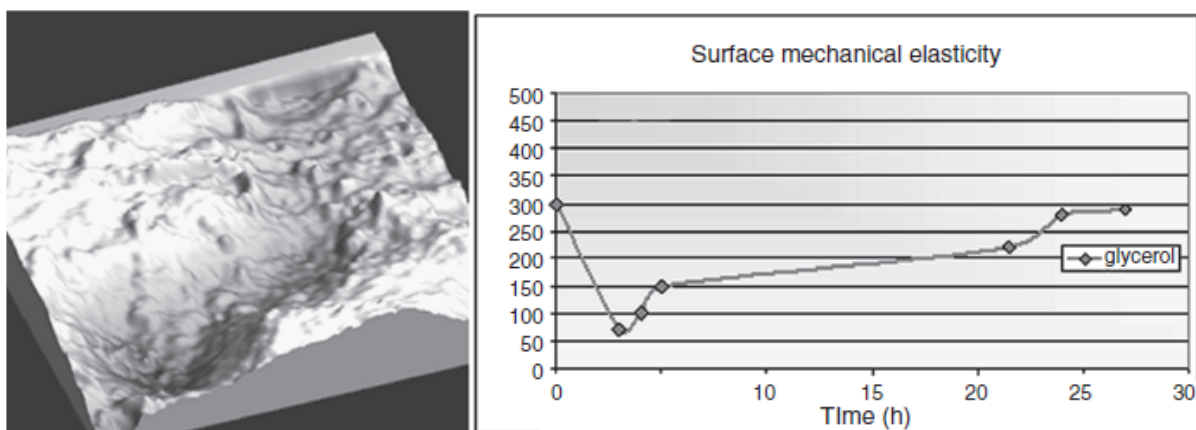


Fig. GL4. Example of an AFM image (top) obtained at the surface of isolated stratum corneum. Measured Young's modulus ($\times 10^6$ Pa) versus time (hours) after applying a common hydrating agent (glycerol). By comparing images and mechanics, the action mechanism absorption versus film forming can be discerned (see text).

Glycerol (7% wt) is applied on stratum corneum. The extreme surface elasticity is then measured. The glycerol forms a smooth layer at the surface that penetrates slowly. The original surface topography is recuperated after 27 heures. we performed AFM nanoindentations at different times during this process. The elastic modulus of the SC

measured ($\sim 2.5 \cdot 10^8$ Pa) decreases at first ($\sim 1.0 \cdot 10^8$ Pa) after application, but then the modulus recovers slowly as the glycerol penetrates further inside the stratum.

Evidently this technique remains very local and we have not looked at the whole stratum corneum itself but its surface. Other tests, more currently used can probe at the meso and macroscale providing a complementary description.

Nevertheless, the possibility of mapping the mechanical properties of cells, and corneocytes in particular, at this detail is impressive and will help us understand in the future more about the different contribution of the corneocyte's membrane, desmosomes, etc. on the overall mechanical properties. In dermatology, there are many possible future applications of these AFM techniques in vitro, that will be helpful for understanding different abnormalities of the skin, in particular desquamation disorders.

MEASURING IN VITRO BIOMECHANICAL PROPERTIES OF THE STRATUM CORNEUM AND THE EFFECT OF HYDRATION

INTRODUCTION

The stratum corneum is a composite material made of corneocytes, intercellular lipids, corneodesmosomes and other intercellular proteoglycans. It protects our body from harsh environmental factors and mechanical insults. In the meantime, its capacity to deform and its softness are responsible for the comfort of the skin.

Many cosmetic treatments rely on the deposition and transfer of materials onto the skin surface to repair or improve its properties. Knowledge of the subtle changes occurring in the stratum corneum is essential to develop new skin care products and to find new targets for active molecules.

Several questions may be asked:

- What is the role of the different constituents on the mechanical properties of the stratum corneum?
- What is the interaction between water and the stratum corneum, between water and each of these constituents?
- Hydration induces flexibility of the stratum corneum. Are there other plasticization mechanisms to soften the stratum corneum?
- In order to take into account the anisotropy of the stratum corneum, what techniques can be used to measure the mechanical properties of the stratum corneum?

In this chapter, we will try to answer partly to these questions. Several aspects will be evoked:

- a description of the macroscopic approaches developed to describe the biomechanical behavior of the stratum corneum and the effect of hydration and cosmetic ingredients,
- a review of the studies performed at a more local scale to elucidate the role of the different constituents,
- and at last the first results obtained by a new mechanical approach by nano-indentation.

MACROSCOPIC MECHANICAL PROPERTIES: A STATE OF THE ART

MACROSCOPIC MECHANICAL APPROACHES

Stress relaxation tests have been performed on human stratum conditioned in various physicochemical environments [19-24]. A typical stress-strain curve for a stratum corneum conditioned in water at 25°C for one hour reveals three distinct behaviour regions separated by inflections at approximately 25% and 125% extension. This mechanical behaviour is qualitatively similar to that found for wool and human hair stretched under similar conditions. Nevertheless differences are pointed out and discussed [19,28]. The determination of the static modulus remains difficult, as it is explained by Park and Baddiel [19,20]. Over the

increasing 0 to 100% HR range, untreated stratum corneum breaking strength decreased 85%, while the work of fracture increased 600%. Elongation at fracture increased from 20% at 0% HR to 190% at 100% HR [21]. The effect of relative humidity, over the range 30-100% on the elastic modulus is dramatic, the value changing from 2 GPa at 30%HR to 3 MPa at 100% HR [19]. These figures illustrate the remarkable property of long-range elasticity exhibited by stratum corneum and the great effect of adsorbed water on its deformation behavior.

The load-deformation behaviour of SC was also investigated using a pure shear specimen geometry. The tissue displayed non-linear load-deformation behaviour and stress relaxation, although the extensibility and amount of stress relaxation was considerably less than that show by other soft connective tissue [22].

Rheological models have been used to forecast the results of constant strain rate tensile tests. The linear viscoelastic behavior which is the most widely used in static tensile tests does not apply to stratum corneum. The nonlinear model gives good results [23].

Several dynamic measuring instruments have been used or developed for measuring the changes of the mechanical properties of the stratum corneum with its water content or with time when cosmetic ingredients are applied.

Several authors have developed especially dynamic measuring instrument to estimate the effect of treatments on the mechanical properties of stratum corneum. The determination of the elastic and viscous dynamic moduli are more precise and easier than the static modulus [28]. The softening effect is usually evaluated by the ratio of dynamic elastic modulus E'_t (at time t after application of a test solution) / $E'_{t=0}$ (for the non treated stratum corneum) [25-28].

INFLUENCE OF RELATIVE HUMIDITY

It is well known that the flexibility of the stratum corneum depends on its water content [21,23,25,26]. J.L. Leveque, L. Rasseneur et al. have extensively studied water-keratin

interaction in human stratum corneum. Elastic modulus of human stratum corneum has been recorded at various relative humidities (RH) as well as the water content and the water interaction energy. The binding state of water molecules with the keratin highly influences the viscoelastic properties of the stratum. Two different processes are described. At $RH < 60\%$ the water would condense in a single layer on active hydrophilic sites, with a high interaction energy (58kJ/mole). During this first stage of hydration the modulus of elasticity varies greatly: a decrease of more than 10% per 1% of sorbed water. Above 60%RH the decrease in molar energy indicates that the primary sites are saturated and that interactions are reduced. The modulus decreases by about 1% per percentage of sorbed water [25].

Concerning the role of the different constituents on the water retention capacity of the stratum corneum, experiments have been performed on non treated stratum corneum versus samples without lipids (by solvent extraction) and samples without lipids and NMF (by solvent extraction and water washed) [19,20,25]. It appears that:

- the water soluble substances (so called NMF i.e. “natural moisturizing factors”) strongly influence the equilibrium rate of the stratum corneum with the environment. They would help water diffusion towards the cellular keratins sites, through the barrier formed by the intercellular spaces and the membrane of corneocytes. They give a substantial water-absorbing power to the stratum.
- the intercellular lipids appear to play an important role in protecting the hydrosoluble substances. In the low humidity range, the lipids could hide some bonding sites. When the relative humidity reaches 65%RH, more water penetrates the structure when lipids are removed [25].

INFLUENCE OF COSMETIC INGREDIENTS

The lack of reproducibility between samples of stratum corneum has led many authors to develop mechanical techniques where the same sample is used before and after treatment.

A. Rochefort et al. have measured the initial slope on the stress-strain curve to evaluate the effect of cosmetic products [24]. It is shown that water treatment is not able to durably soften the stratum corneum, whereas emollient is able to act *in vitro*, where the large pool of water in the deeper tissue layers is missing. The authors invoke a mechanism by which the surface topography becomes easier to deform after treatment. The part played by the contribution of the deformation of the surface undulations might be more important than suspected as far as the small forces are concerned [24].

M. Takahashi et al. have used an especially dynamic measuring instrument to estimate the change of skin-softening effects of humectants with time, after topical application. They demonstrated that [26,27]:

- both polar and non-polar oils which are widely used in cosmetic products did not soften the stratum corneum,
- the effects of aqueous surfactants solutions were characterized by an increased of elasticity after evaporation of water,
- the plasticizing abilities of humectants depended on their water-holding capacities,
- non-hygroscopic hydroxy acids appear to soften stratum corneum by a plasticizing effect of keratin chains, without increasing the water content in stratum corneum.

The effect of thioglycolic acid (TA) and salicylic acid (SA) on the stratum corneum was also studied by L. Rasseneur et al by means of mechanical measurements and electron microscopy.

TA markedly reduces the elastic modulus. Electron microscopy shows that the corneocyte envelope of superficial corneocytes is fully degraded. On the contrary, SA treatment increases the elastic modulus without any noticeable changes on corneocytes. The microscopic observations show the preferential effect of SA on the intercorneocytes spaces. It

is proposed that SA degrades the intercellular proteins and so leads to a water extraction of the soluble NMFs and a dehydration of the sample [28].

These studies show the needs to demonstrate the relationships between the SC constituents and the visco-elastic properties of the stratum corneum.

ROLE OF THE DIFFERENT CONSTITUENTS ON THE BIOMECHANICAL PROPERTIES OF THE STRATUM CORNEUM: STATE OF THE ART

Whereas several studies have been performed to elucidate the role of the different constituents on the mechanical properties of the stratum corneum, many questions are still open.

Since the “brick and mortar model”, in which intercellular lipids were described as playing an important role in stratum corneum cohesion, it has been fully recognized that the corneodesmosomes complexes play a crucial role in cohesion of this tissue and the desquamation process. Different desmosomal glycoproteins (desmogleins, desmocollins, mainly *dsg1* and *dsc1*, belonging to the cadherin superfamily) and corneodesmosin, a specific adhesive protein of the SC, are believed to be key adhesion molecules responsible for corneocytes cohesion. These corneodesmosomal proteins are linked to keratin filaments via the membrane proteins of the corneodesmosomal plate, assuring continuously the strain transfer all over the stratum corneum.

The precise relationship between lipids and stratum corneum elasticity remains controversial. For Middleton et al. lipids extraction had no consistent effect on stratum corneum extensibility [29], and for Park and Baddiel it did not influence its elastic properties [19,20]. In contrast, JL Leveque et al. suggested that lipid play a slightly role in plasticizing the stratum corneum [25].

There are fewer studies on the morphological evidence for changes in the structure of the stratum corneum constituents during mechanical extension or mechanical delamination.

MORPHOLOGICAL CHANGES DURING MECHANICAL EXTENSION

AV. Rawlings, A. Watkinson et al. have examined in detail changes in the structure of the stratum corneum, i.e. intercellular lipids and corneodesmosomes during in vitro linear extension studies. Ultrastructural changes have been investigated by electron microscopy. Initially, at low extensions of SC (2% extension) the structure of the intercellular bilayers lipids appeared normal; during further extension (5% extension) their membrane became disrupted, and with continued extension (8% extension) they became progressively disorganized. Desmosomes, by comparison, were more resilient structures, only being perturbed after large extensions during which intercorneocyte desmosomal links were observed to rupture (break?) just before the complete fracture of the tissue [30].

J.L. Leveque, P. Hallegot et al. have performed the same kind of experiments, combining transmission electron microscopy and X-ray diffraction. Traction was conducted in water solution. The most prominent change induced in human SC maintained at 60% extension is the detachment of the lipid multilayers from one of the adjacent corneocytes. Corneosomes were detached from the corneocytes envelope and in some instances ruptured. Thus, the binding cohesion force relative to corneocytes-corneosome junction appeared weaker than the intrinsic resistance of the corneocytes and corneosomes themselves. The supramolecular organization of intercellular lipids in the form of multilayers was found globally unmodified by X-Ray analysis [31].

The effect of mechanical stress on the barrier function is also controversial. For Leveque et al., submitting human SC to an extension force up to 20% elongation does not significantly alter the barrier function [31]. For Rawlings et al. stratum corneum lipids are sufficiently fluid to maintain barrier function during small extensions of the skin surface. However, following

large extensions (> 8% extension) the combination of desmosomal rupture and the lipids structural changes could lead to perturbed water barrier function [30].

MICROSTRUCTURAL PROPERTIES DURING DELAMINATION

Surprisingly few studies have been performed to study the mechanical properties of the stratum in the direction normal to the skin surface.

Controlled failure tests were carried out in the pure shear test specimen by Koutroupi et al. The fracture surface energy of stratum corneum has a mean value of 3,6 kJ/m² which is comparable to the tougher synthetic polymers [22].

An in vitro mechanics approach has been developed by KS Wu, RH Dauskardt et al. to examine the SC intercellular delamination energy as a function of temperature, moisture and chemical treatments. Resulting failure surface morphologies were examined by scanning electron microscopy [32,33,35]. The delamination energy would be dominated by the cohesive properties of the intercellular boundaries of the SC rather than plastic deformation. Increased hydration in concert with increasing temperature seems to play a key role in reducing the delamination energy value, with the most significant changes occurring at 100% RH conditioned SC. The observed decrease in delamination energy was associated with a reduction in SC cohesive strength. Lipid disordering would be not enough to weaken the intercellular structure significantly. Intercellular lipid extraction by chloroform – ethanol solution significantly increases delamination energies, as explained by subsequent interaction of remaining lipids covalently bounded to corneocytes envelope. Other remaining constituents, such as glycoproteins and HSPG, could be on the origin of this interaction. Little hydration dependence was observed on delipidized samples [32,35].

Concerning the effect of the corneodesmosome protein linkages between cells, it is likely that the delamination energies are not correlated highly with expected corneosome cohesive

contribution [35]. Nevertheless measured delamination energies were found to increase from $\sim 3\text{J/m}^2$ near the surface to 15J/m^2 for the inner layers of the tissue [33]. This result would be in agreement with the studies of Chapman et al [34]. This would be correlated with the natural increase in corneodesmosomes toward the inner components of the SC.

The SC delamination energy was found to be relatively insensitive to pH and 1% SDS treatments. More elevated values were observed for SC treated with a 10% SDS solution, for the same reason as delipidized samples.

Initial measurements of modulus reveal that SC stiffness in this mode is much lower than reported in-plane. A simple bricks and mortar model requires refinement to explain the highly anisotropic mechanical behavior exhibited by SC [35]. The mechanisms causing the differences may be attributed to additional SC micro-structure. Intermediate filaments such as keratin fibers are linked to corneodesmosomes, which bridge the intercellular space to connect adjacent cells. The structural orientation and possible alignment of these keratin fibers, which is up to now controversial, would have a strong impact on this mechanical anisotropy. These corneodesmosomes may facilitate the transmission of tensile forces between cells leading to the greater stiffness and higher fracture energies observed in the in-plane orientation [32].

The increased macromolecular mobility of these fibers with increasing hydration represents one of the reasonable mechanism leading to decreased modulus with increased fiber sliding. The increased macromolecular mobility of the corneodesmosomes components with hydration or plasticizing effect would be another reasonable mechanism for the origin of SC softening effect.

ROLE OF THE DIFFERENT CONSTITUENTS ON THE BIOMECHANICAL PROPERTIES OF THE STRATUM CORNEUM : A NEW APPROACH

The compression mechanical properties of SC have been explored by different teams. Y Yuan and R Verma have used an atomic force microscope (AFM) as well as a Triboscope nano-indentor and a nano-DMA (Hysitron) to measure the visco-elastic moduli (E' and E'') at the micro-level. Measurements have been conducted on isolated dry and wet stratum corneum at varying depths [36]. The elastic moduli values obtained with a purely elastic model are on the order of 100 to 10MPa for dry and wet SC, respectively. The measured $\tan\delta$ is seen to increase from ~ 0.1 to 0.25. An apparent modulus variation with indentation depth is observed. The origin of this behavior is not understood.

THE STUDY OF THE ROLE OF THE DIFFERENT CONSTITUENTS BY A SUB-MICRON NANO-INDENTATION APPROACH

The focus of the present study was to measure the mechanical properties of the stratum corneum at a sub-micron length scale, in order to investigate the effect of humidity, cosmetic treatments and to specify the role of the different constituents. A nano-indentation technique was used [37,38].

Effect of humidity:

The same sample was used at 3 humidity rates 25%, 50%, 70% and immersed in distilled water. The results are reported in Figure 1.

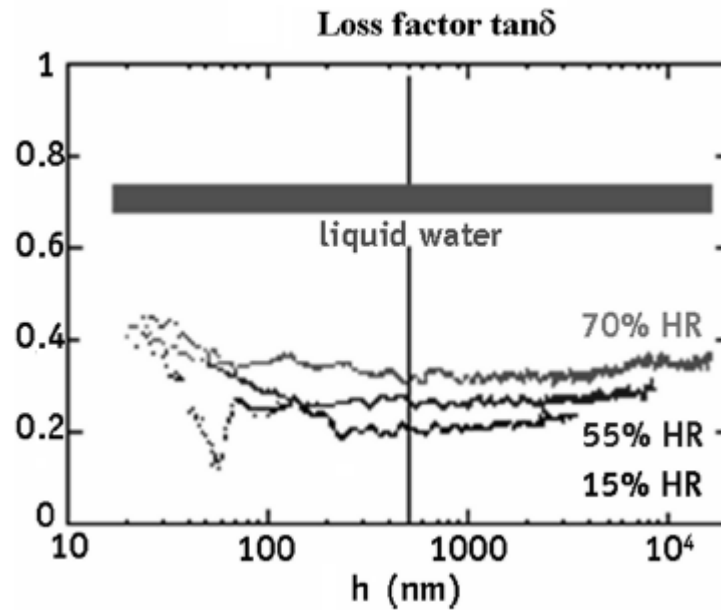


Figure 1: loss factor ($\tan\delta$) of SC at different humidity rates

Nanoindentation test at controlled temperature and humidity: nanoindentation tests were performed with a MTS XP Nanoindenter using the continuous stiffness measurement method to determine the stratum corneum's viscoelastoplastic properties. This method consists in superimposing a small displacement oscillation at a given frequency ($a=5\text{nm}$) during the indentation test. The frequency of the added harmonic vibration is 32Hz. The apparatus is inside a climatic chamber. Reduced Young's modulus and loss factor as a function of penetration depth are measured continuously by the simultaneous measurement of the normal load and the contact stiffness.

A reduction of reduced young modulus and an increase of the loss factor is found as a function of humidity. A periodic response is recorded, which could be linked to the successive contribution of the intercellular components. Our hypothesis is that the loss factor $\tan\delta$ is closely related to the intercellular spaces macromolecular mobility and viscosity.

A gradient of mechanical properties depending on indentation plastic depth is observed.

Role of the different constituents

The results are reported in figures 2.

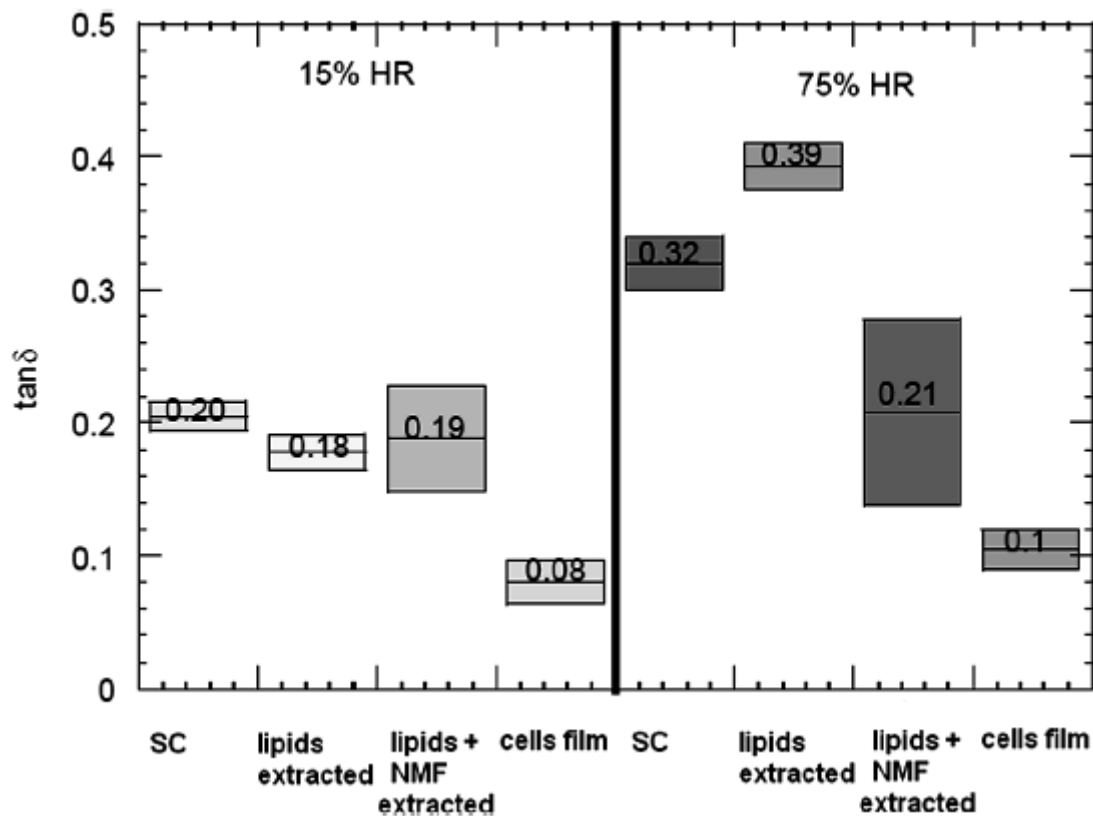


Figure 2: Loss factor evolution as a function of treatments at 2 humidity rates

Stratum corneum is issued from abdominal plastic surgery. In order to investigate the role of the different constituents, several treatments were applied on the *stratum corneum*: delipidation (chloroforme/methanol), NMF removal (delipidation + water rinsing). A « *corneocytes film* », made of isolated *corneocytes* stacked up, was also constituted by evaporation of a *corneocytes* dispersion (*corneodesmosomes* and soluble proteins removal by EDTA + TritonX100).

- Role of intercellular lipids:

At 70%HR, without lipids, the loss factor is slightly higher than the one of the natural *stratum corneum*. Water would easily reach on hydrophylic intercellular spaces and plastification would be more important. Viscosity of intercellular macromolecules (*corneodesmosomes* or other proteoglycanes) is increased.

- Role of NMF:

At 70%HR, without NMF and other soluble intra and extracellular proteins, stratum corneum is stiffer and less dissipative than delipidated SC. The water diffusion towards the intracellular keratin sites is restricted.

- Role of corneodesmosomes:

At 15 and 70%HR, the elastic modulus of the « corneocytes » film is much higher than the one of the natural stratum corneum. Loss factor is much smaller.

The « corneocytes » film is stiffer. The “macromolecular mobility” or “sliding” of the junctions between the constitutive elements (corneocytes) is very difficult.

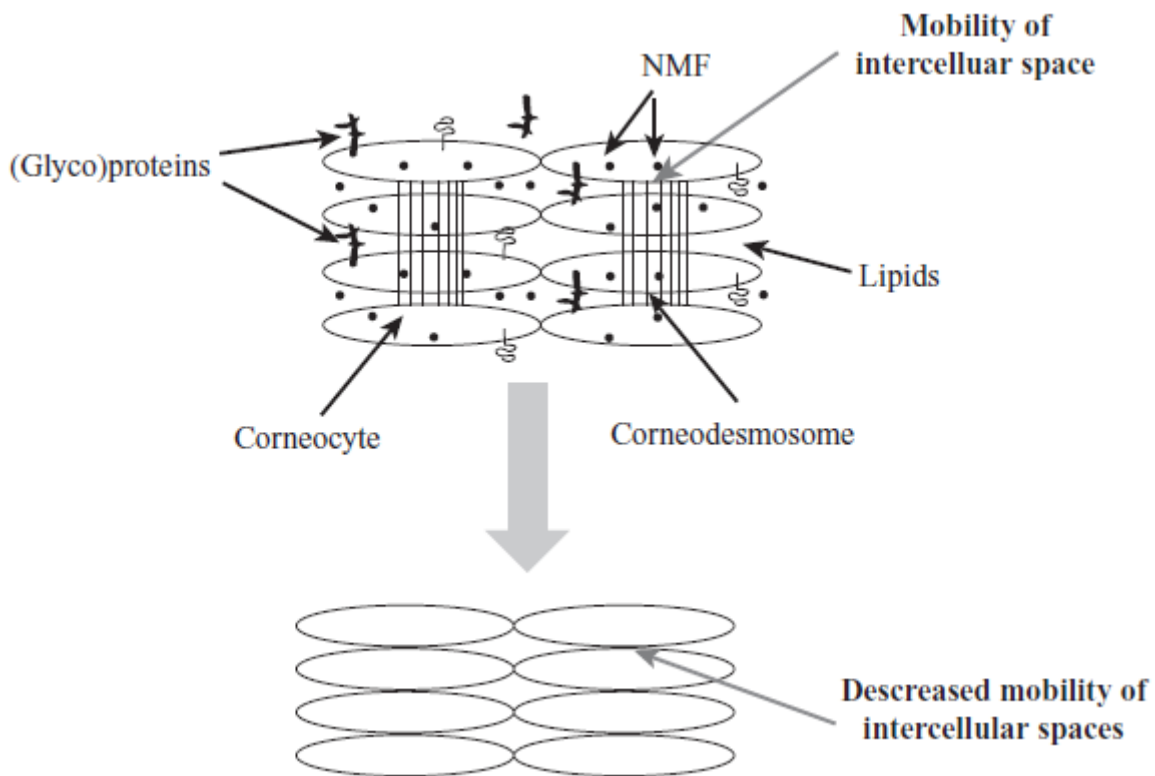


Figure 3: schematic presentation of the normal SC versus the “corneocytes” film.

These results demonstrate the importance of the intercellular spaces on the mechanical behavior of stratum corneum. The flexibility of SC would mainly be related to the corneocytes connexions and the lubrication of these intercellular spaces.

Effect of moisturizing products:

The loss factor values are reported for a depth penetration of 1 μ m (Figure 4).

Regarding the 3 moisturizers, the modulus of the stratum corneum is reduced and the loss factor is increased after treatment. This well-known plasticization effect was induced by an increase of the water retention inside the stratum corneum. The particular strong effect induced by the urea treatment would be related to a strong interaction (plasticizing effect) of urea with the proteinic constituents, particularly those located in the intercellular spaces.

The surface mechanical properties may also be measured. The mechanical values are reported for a depth penetration of 100nm (Figure 4). Concerning glycerol and urea, the surface modulus was decreased and an adhesive component was measured for glycerol.

As for the amphiphilic polymer Aristoflex LNC, the surface modulus was increased, related to the formation of a surface polymer film and no adhesive component was measured.

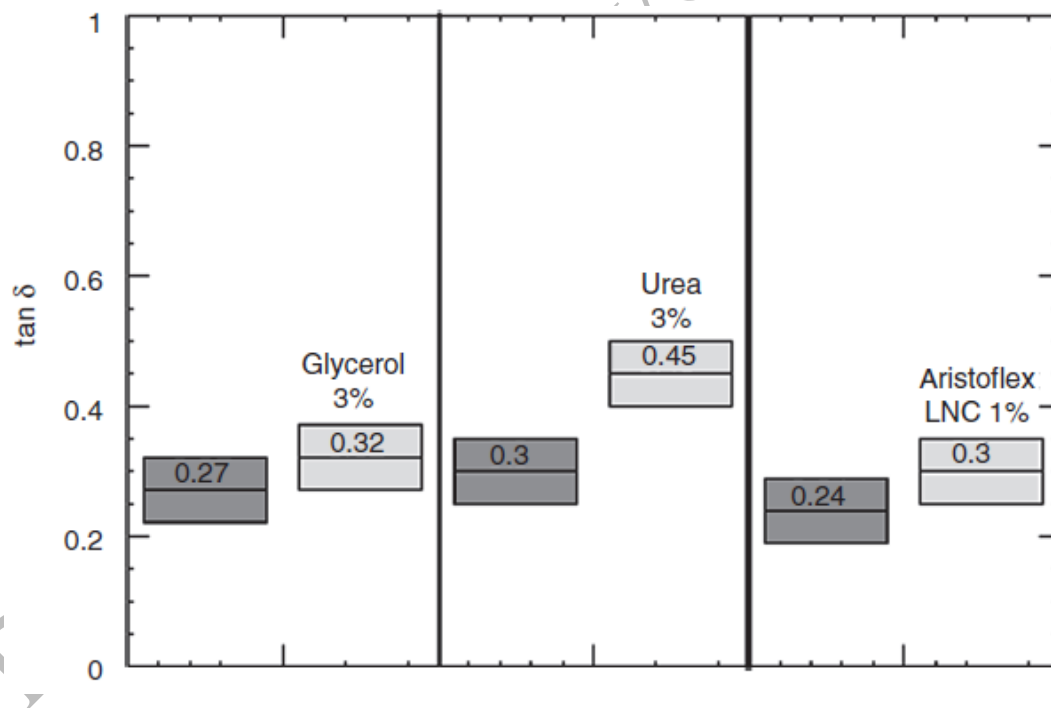


Figure 4: Loss factor $\tan \delta$ (1- μ m depth) at 50% HR of SC treated by aqueous solution of glycerol 3%, aqueous solution of urea 3%, and aqueous solution of polymer Aristoflex LNC 1% (Clariant), respectively

Conclusion:

With the nano-indentation technique, it is possible to measure both mass and surface mechanical properties of the stratum corneum. It allows to investigate the effect of humidity or of actives molecules and to specify the role of the different constituents.

The proposed hypothesis is that the softness and the flexibility of stratum corneum are mainly related to the corneocytes connexions, their viscosity and the lubrication of these intercellular spaces components. The strong effect of urea treatment on the loss factor value would be an illustration of this hypothesis. Further studies using specific treatments and different relative moistures have to be conducted to improve this hypothesis.

The results contribute also to distinguish different modes of action of moisturizing molecules: hydration effect of glycerol, plasticization effect of urea with a strong interaction with proteinic constituents and a new moisturizing mechanism by surface structuration of an amphiphilic polymer.

IN VIVO MECHANICAL PROPERTIES OF THE STRATUM CORNEUM

In this section we discuss about in vivo mechanical properties of the stratum corneum and its contribution to the global behavior of the skin, view as a multi-layer organ. In particular, we review the principal methods that have been employed by the scientific community to assess in vivo the mechanical behavior of the skin and their applicability to characterize the stratum corneum. We then focus on the influence of this layer on the wrinkling capacity of the skin.

CURRENT RESEARCH

Much research has focused on the in vivo mechanical characterization of the skin, as reviewed by Diridollou et al. [39]. The techniques the most frequently employed are suction [39-41],

torsion [42-44], tensile tests [45-47] and wave propagation [48-50]. All these methods measure the *global* response of the skin to the applied load.

Some author tried to identify the uppermost skin layer contribution by small aperture suction experiments [40], small annular size torsion test [44] or gas bearing electrodynamicometer [45]. They started from the principle that the smaller the loaded area and the lower the loads are the more superficial the skin deformations are and, as a consequence, the more important the stratum corneum contribution is in the resulting skin mechanical response. However, as the skin is made up of three main layers, a 50 μm -thick epidermis, a 1 mm-thick dermis and a several millimeters-thick hypodermis, all these approaches would prove difficult in terms of recovering the intrinsic mechanical properties of each layer independently.

More recently, dynamic elastography [51] has been suggested as a new *local* non-invasive method for skin mechanical properties measurements. This approach has been proven to be accurate to investigate dermis and hypodermis local Young's modulus. However, no studies relate on the application of this technique to thinner skin layers like stratum corneum or living epidermis.

In the last few years, skin mechanical parameter identification through inverse analysis has been proposed as a new effective method to differentiate and characterize the layer behavior by coupling a biomechanical numerical model of the skin with a mechanical experimental test. In this approach, a model of skin, containing as an input the mechanical parameters to be determined, is employed to simulate the experimental test. These parameters are then identified by minimizing a fitness function describing the discrepancy between measured and simulated data. For a given biomechanical model, the choice of the suitable experimental test is of paramount importance, since on it relies the possibility to determine the unknown parameters in a unique and robust way. In the literature, several models with different degrees of complexity and associated to different experimental tests have been considered. Single-

layer models coupled with suction [52], indentation [53] or tensile test [54] have been proposed to assess average skin mechanical parameters. The two-layer model proposed by Hendricks et al. [55], coupled with suction experiments at different apertures, allows to determine the mechanical properties of the reticular dermis and of a mixture of papillary dermis and living epidermis. A three-layer model associated to indentation has been proposed by Pailler-Mattei et al. [56] to measure the Young's modulus of dermis, hypodermis and muscle. Tran et al. [57] developed a four-layer model coupled to an indentation test, which allows to extract the mechanical parameters of epidermis, dermis, hypodermis and muscle.

Therefore, to our knowledge, no published data refer to experimental or inverse analysis approaches to measure *in vivo* the mechanical properties of stratum corneum. As a consequence, no results are available about the *in vivo* effects of the skin hydration on the mechanical properties of the stratum corneum.

Several studies focused on the hydration effects on the behavior of the whole skin under different mechanical loads, such as torsion, suction or traction, however none of them analyzed the hydration effects on wrinkling capacity of the skin. Wrinkling has rather been studied in association with skin transformations due to age, in particular in relation to the mechanical and morphological evolutions of the different layers [6,58,59]. In [6] the authors presented a new device, the Densiscore, which has been developed to assess the wrinkling capacity of the skin in a reproducible way, then providing a more objective procedure to estimate folding with respect to usual clinical procedures. They classified the degree of wrinkling by assigning a score from 1 to 6, according to the fold characteristic length λ and amplitude, where score 1 corresponds to the subjects without wrinkles, score 6 corresponds to the subjects with large and pronounced wrinkles, with λ of a few millimeters. The authors also showed that the degree of wrinkling correlates well with age. In [58] the authors developed a three-layer mechanical model of the skin in order to interpret the increased skin wrinkling

with age in terms of evolution of the mechanical properties of the different skin layers. They concluded that both stiffening of the stratum corneum and morphological changes of living epidermis and papillary dermis could explain the observed clinical differences between young and aged skin wrinkling. In [59] the authors discussed aging and wrinkling in terms of a five-layer mechanical model of the skin, and showed that a sudden apparition of wrinkles at a certain age was obtained as a consequence of stiffening of stratum corneum and papillary dermis, along with stiffening and thinning of the living epidermis.

Therefore, in the next section, we will focus on the effects of hydration on skin wrinkling, paying special attention to the role of the stratum corneum.

INFLUENCE OF STRATUM CORNEUM ON THE WRINKLING CAPACITIES OF THE SKIN

Wrinkles emerge and become more pronounced with age, they depend on the nature of the skin and muscle contraction. Two kinds of wrinkles are usually considered: expressive wrinkles and wrinkles due to age. The first appear around specific body sites during muscle contraction. They disappear after removal of the muscle mechanical load. Repetition of skin folding on the same site would progressively give rise to permanent wrinkles, which first appear on the face around 30-40 years old, especially around eyes. In this section, we will focus on the first kind of wrinkles.

With the help of a mechanical model of the skin, we will discuss how wrinkling depends on the mechanical properties of the stratum corneum, and how their modifications after hydration affect the wrinkle morphology.

Materials and methods

We employed a three-layer mechanical model, representing stratum corneum, a mixture of living epidermis and papillary dermis (referred in the following to as middle layer) and

reticular dermis. Each layer was considered as elastic, incompressible, isotropic and homogeneous. The model equations are discretized and solved by a finite element approach, in the framework of a two-dimensional plane stress approach. Such approach was proved to be accurate enough to successfully explain clinical observations about the evolution of the skin wrinkling with age [58].

The model was employed to simulate the behavior of the skin under in-plane compression, as in the Densiscore [6] experience. The wrinkling is obtained for loads higher than a critical value, a phenomenon known as buckling, and the characteristic length λ of the folds developed at the critical value was calculated.

Results and discussion

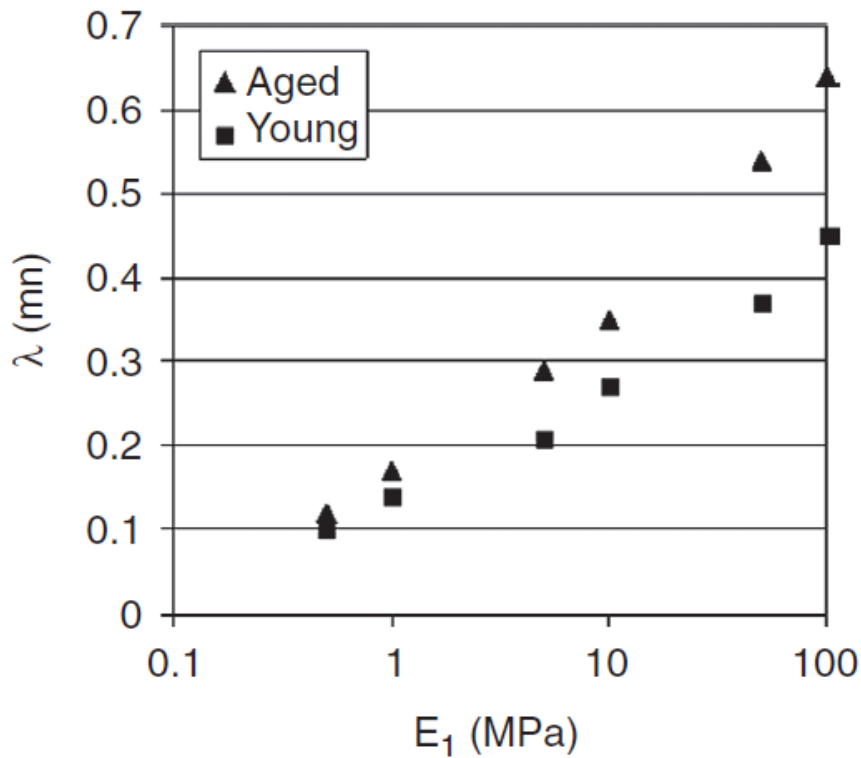


Fig. 5. Characteristic length λ of the folds in simulated wrinkled skin as a function of the stratum corneum Young's modulus E_1 for both a young and an aged subjects. After [21], the simulations were performed using the following parameters. Young subject: $t_1=15 \mu\text{m}$, $t_2=50 \mu\text{m}$ and $t_3=1.235 \text{ mm}$ for stratum corneum, middle layer and reticular dermis thicknesses, $E_2=0.05 \text{ MPa}$ and $E_3=0.6 \text{ MPa}$ for middle layer and reticular dermis Young's modulus. Aged subject: $t_1=15 \mu\text{m}$, $t_2=200 \mu\text{m}$, $t_3=1.085 \text{ mm}$, $E_2=0.05 \text{ MPa}$ and $E_3=1 \text{ MPa}$.

The Fig. 1 shows the characteristic length λ of the folds in simulated wrinkled skin as a function of the stratum corneum Young's modulus E_1 . On the same figure, a comparison is made between a young and an aged subject, whose mechanical and structural parameters were taken from [58]. The results indicate that, for both young and aged subjects, the characteristic length decreases by decreasing the stratum corneum Young's modulus. This indicates that after skin hydration, the wrinkles become smaller and more numerous, thereby approaching a younger skin. This behavior is due to a decreased flexion rigidity of stratum corneum due to its decreased Young's modulus, which makes shorter length and smaller amplitude folds energetically more favorable.

The data also suggest that the impact of the stratum corneum Young's modulus on the skin wrinkling is smaller than the effect of the middle layer's thickness or the dermis Young's modulus, according to [59]. In fact, by comparing the two curves, we notice that the difference between the values for the young and the aged subjects, at E_1 fixed, is comparable to the difference, on a given curve, between two values of E_1 . However, as can be deduced from the values of the parameters reported in the figure caption, the differences between young and aged middle layer's thickness and dermis Young's modulus are smaller than the variations of E_1 , which spans several order of magnitude. The results therefore demonstrate that the simple softening of the stratum corneum has a limited effect on the reduction of the wrinkles.

Conclusions

Up to now, no studies have been reported on the measurement of the stratum corneum mechanical properties in vivo and their modifications due to hydration, for instance after the utilization of a moisturizer. Rather, some studies discuss the impact of hydration on the whole skin behavior. In particular, we focus on the role of the stratum corneum mechanical

properties on the wrinkling capacity of the skin and its evolution after the horny layer softening due to hydration.

Due to the lack of specific information on in vivo properties of stratum corneum, in vitro published data at different length scales still are our primary source of information about its mechanical behavior.

GENERAL CONCLUSION

This chapter has underlined the remarkable property of long-range elasticity exhibited by stratum corneum and the great effect of adsorbed water on its deformation behavior.

Regarding the role of the different components of the SC on its mechanical properties, their interaction with water should also be studied.

The intercellular lipids themselves would have little influence on the softening effect of the SC. They play an important role in protecting the hydrosoluble substances and in low humidity range they could hide some binding sites.

The NMFs strongly influence water diffusion towards the proteinic and the keratin sites.

The flexibility of SC would mainly be related to the corneocytes connexions and to the macromolecular mobility of the corneodesmosomes proteins.

The influence of the cell envelope has hardly been studied.

The cell envelope / corneodesmosome / keratin filaments system is the only continuous phase pervading the entire cell structure of the stratum corneum and is so likely to be the load bearing component [20]. It is suggested that the cell membrane protein might be as important as the keratin or the corneodesmosomes proteins in controlling the mechanical properties of the stratum corneum. This would mean that another rheologically active material could be the cell envelope system. The concept that the mechanical strength of the corneum resides in the cell membrane system is supported by the fact that the cell membrane protein has much higher cystine content (x3-4) than the keratins filaments, leading to more permanent (with regard to disruption by water) disulphide cross-links in the membrane [20].

All these in vitro studies still are our primary source of information about stratum corneum mechanical behavior, because, up to now, no studies have been reported on the direct measurement of the stratum corneum mechanical properties in vivo and their modifications due an external agent.

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