Application of atomic force microscopy in skin related research

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The detailed knowledge about skin structure is of great interest especially for scientists involved in the development of skin equivalents. A better understanding of biomechanical properties of outer skin layer can be useful in pharmacy for creating models for wound healing, medicine and cosmetic industry. So far different methods such as electron microscopy, confocal scanning laser microscopy, confocal Raman microscopy and ultrasound microscopy have been applied in skin related research. However, recently atomic force microscopy (AFM) has been also used as a new technique for skin characterization. AFM enables to image the surface properties of biological materials with a very high resolution and it can provide unique information regarding roughness, stiffness and elasticity of studied sample. Additionally, it can be used to quantify the mechanical properties of skin at nanoscale resolution in the native state. The aim of this chapter is to review recent studies concerning the application of atomic force microscopy in skin related research. The article provides a general overview of AFM and presents experimental aspects that are crucial to apply this technique in skin studies.

Keywords: stratum corneum; atomic force microscopy; skin; mechanical properties; nanomechanics

1. Introduction

Skin is a barrier that protects the organism from external factors, prevents excessive water loss and regulates temperature of the body. The knowledge about human skin structure and tribology and also its mechanical properties is of great interest to medicine, pharmacy and cosmetic sciences. So far many different techniques such as electron microscopy [1], confocal scanning laser microscopy [2,3], ultrasound microscopy [4], confocal Raman microscopy [5], electro-impedance techniques [6] have been used to analyze skin. Recently atomic force microscopy (AFM) has also been applied to study skin properties. The review provides information on the lately published literature regarding the application of AFM in skin related research.

2. Atomic force microscopy – general technique description

The atomic force microscopy belongs to the family of the scanning probe microscopes that are based on different types of interactions such as magnetic, electrostatic, van der Waals interactions [7]. AFM enables to characterize the samples at nano- and sub-nanometer scale in their native conditions. This technique is based on the detection of forces acting between the sample surface and tip that is attached to cantilever [8,9]. During the measurement the surface of the sample is scanned and the deflection of the cantilever is recorded with sub-nanometer precision. In this way the sample surface topography is obtained. Atomic force microscopy enables to quantitatively assess the nanomechanical properties of the surface by determining relations between the sample and probe distance and force between them [10]. AFM technique provides specific information about the sample. It enables to measure the surface roughness. Additionally, it can be used to detect surface stickiness, elasticity (rigidity), adhesion properties and friction between AFM probe [11-13].

The atomic force microscope can operate in different modes such as contact mode, non-contact mode and tapping mode. In contact mode the tip slides over the surface and deflects according to the profile of the sample. The tip is in contact with the sample while scanning. The experiment in contact mode could be performed in two different ways by using the constant force mode and the constant height mode. In the first mentioned mode, a feedback loop is applied to shift a sample or the tip up and down and keep its deflection constant [14]. As the result the surface topography of sample is obtained. During the measurements performed in the constant height mode a height remains constant but the forces change. The cantilever deflection is estimated directly. In order to determine the distance from the surface the deflection force on the tip is applied. This mode is suitable for quick examination of the samples with small height difference [15]. In non-contact mode the tip is forced at high frequency to oscillate above the surface [16]. In this mode even weak forces such as van der Waals or electrostatic forces could be detected. In tapping mode the tip makes a transient contact with the sample at the bottom of its swing. The cantilever oscillates with larger amplitude than in non-contact mode. The main advantage of AFM is that the native untreated samples could be analyzed. This technique allows imaging without any denaturation process of the sample. The measurements by atomic force microscopy can be performed both in air and under aqueous conditions; therefore this technique could be an ideal tool for analysis of the complex biological samples such as skin.

3. Skin structure

Skin can be considered as a multi-layer complex biomaterial, which functions include barrier protection from external factors, sensory reception and heat regulation. The skin structure and its complexity are of great interest for scientist involved in developing synthetic skin equivalents. Skin is a layered natural biomaterial and also organ that comprises of subcutis, dermis and epidermis [17,18]. The subcutis that is localized under the dermis consists of the fat cells with collagen partition. The dermis, which lies below the epidermis, is composed of collagen and elastic fibers and ground substance of proteoglycans and glycoproteins [19]. In dermis there are blood vessels, nerves, hair follicles sweat glands and sebaceous glands. The epidermis consists of four layers such as basal layer, spinous layer, granular layer and stratum corneum (horny layer). Stratum corneum (SC), the outermost layer of skin, consists of corneocytes (keratinized cells) surrounded by a lipid matrix. Stratum corneum consists of 20% of water, part of which is linked with molecules of natural moisturizing factor and lipids in the skin. The SC plays significant role as mechanical, thermal, chemical, photoprotective barrier from external factors. Stratum corneum comes in contact with topical products. Therefore, the detailed knowledge about its properties is very important for development of new skin care formulations.

4. Application of AFM in skin related research

4.1 Skin characterization

Atomic force microscopy was used to analyze the individual corneocytes that are the outermost layer of epidermis [20]. Kashibuchi et al. performed the detailed study on 3D morphology of corneocytes isolated from the upper arm and cheek of volunteers of different ages [20]. The volume, average thickness and real surface area of coneocytes were measured by AFM in the contact mode that enabled to touch and scan the corneocytes surface with a probe. Additional studies were performed on corneocytes obtained from patients with psoriasis and atopic dermatitis. The results proved that depending on the age of volunteers and anatomical location the morphological parameters of corneocytes were different. The 3D characteristics of corneocytes in the diseased skin varied significantly from those of healthy skin. The volume of corneocytes was larger in the case of unhealthy skin. Additionally, Kahibuchu et al. suggested that flatness index of corneocytes, which is calculated by knowing projected area and average thickness could be used to estimate the differentiation speed of the corneocytes. Gaikwad et al. also used AFM to characterize the physical properties of corneocytes [21]. Rigidity, surface corrugation on a submicron level and friction coefficient between a silicon nitride AFM probe and corneocyte surface were assessed. The topography of corneocytes was collected in the contact mode.

To measure rigidity the force-volume mode of operation was applied. This mode gives information about the surface topography and the force curves at the same time.

The AFM was also applied to analyze the structure of stratum corneum at a nanometer scale [22]. Gorzelanny et al. presented the topographic images of stratum corneum collected by tape-stripping. The corneocytes of young and aged volunteers within their native environment of stratum corneum were compared. The AFM measurements were performed by contact mode in air and by tapping mode in fluid. The results showed that single-cell surface area, prominent intercellular gaps and cell surface roughness increased during skin aging. The study demonstrated that AFM in combination with the tape-stripping method could be a powerful tool for high-resolution skin analysis. Fredonnet et al. who studied the mechanical and topographical properties of corneocytes in their native state, proposed new methodology for the tape-stripping method [23]. The corneocytes were collected from the skin surface by 10 successive tape-stripping on the same dermal site. Afterwards the tape was fixed on a glass slide and the individual cells were measured directly without extra treatment. This technique enabled to investigate the surface of corneocyte in direct contact with the underlying cells. Recently Franz et al. introduced a new method to analyze the nanostructures of corneocytes [24]. The tape-stripping was used to collect the samples from healthy individuals and patients with atopic dermatitis. The tapes were analyzed by AFM to obtain topography images. It was demonstrated that nanotopography of corneocytes changed in diseased skin in comparison to corneocytes of healthy individuals. It was suggested that this information could be used as a diagnostic parameter in skin disorder studies.

Geerligs et al. studied the nanomechanical properties of epidermis and stratum corneum. The results demonstrated that both layers had quite similar stiffness as the Young's moduli values were of 1.1 ± 0.2 and 2.6 ± 0.6 MPa, respectively [25]. Achterberg et al. used AFM to determine the Young's elastic modulus E of human dermis at the cell perception level [26]. The results demonstrated that depending on the body area and dermal layer the E of dermis was different. It was observed that the E value tended to increase with age of volunteers.

Additionally, the atomic force microscopy was applied to analyze viscoelastic properties of SC [27]. Furthermore, the mechanical resistance of the outermost skin layer (SC) to deformation was studied at different length scales by AFM [28]. The magnitude of the force required to induce plastic/elastic deformation of SC was assessed by AFM and force measurements. The results showed that the surface of stratum corneum was heterogeneous exhibiting significant roughness at the 100-500 nm.

Kao et al. applied atomic force microscopy based indentation to study 3D mechanical properties of skin at nanoscale [29]. The dermal samples were analyzed in contact mode by a low cantilever set point value. The results demonstrated meaningful variations in the mechanics of skin between various skin layers. Significant differences in the elastic modulus were observed for different anatomical regions.

Recently, Marcott et al. have used a new system to characterize human skin [30]. Atomic force microscopy was combined with infrared laser (AFM-IR) to analyze the stratum corneum. The studies of the normal and delipidized stratum corneum showed that the delipidization process damaged the structure of SC. The delipidized SC had less long-chain CH_2-stretching IR absorption band intensity than the normal SC. These investigations suggest that AFM-IR might be a promising technique to understand the penetration mechanism of different ingredients through stratum corneum.

Hair is actually a modified type of skin, called skin appendages (adnexa). Hair fibres have a structure layers consisting of the cuticle (roof shingles), the cortex, which contains the keratin rod-like bundles and the medulla, a disorganized and open area at the fibre's centre. AFM can provide real-time analysis of human hair morphological changes under different conditions. It was found that pH changed the skin morphology. A kinetic study of hair hydration effect on morphology was determined and it was found that overlapping keratinized cells forming the hair cuticle spread out between 50 and 150% when hydrated, compared to a total shaft diameter change of 10% [31].

4.2 Analysis of scar tissue

Fluid AFM was used to analyze the mechanical and viscoelastic properties of the upper dermis of fully matched human healthy and scar tissue following surgery [32,33]. Higher degree of orientation of collagen fibrils was observed for scar skin, while for normal skin a more random orientation was detected in AFM images. The viscoelasticity of both tissues was analyzed by using dynamic oscillatory technique and by measuring the indentation creep over a fixed time period.

The results proved that the normal skin had greater indentation creep behavior and higher dissipation at a physiologically relevant frequency range in comparison to the scar tissue. Moreover, the dermal scar tissue demonstrated stiffer behavior than the healthy skin. The authors claimed that this favorable dissipative property in normal healthy skin was not remodeled at the nano-level following the wound healing process.

Very narrow-band ultraviolet B (NB-UVB; 311 nm) light is commonly used in the treatment of various skin disorders. It was observed by using AFM that upon NB-UVB, i.e. the most commonly used phototherapy device, the significant rearrangement of the cytoskeleton, causing thinning of microfilaments and their redistribution to the cell periphery was registered [34].

4.3 Studies on drug penetration into skin

In recent years different types of nanomaterials have been applied in topical products. It is believed that they can control the release of active compounds from the formulations [35]. Furthermore, they can protect drug from degradation through particle encapsulation [36]. Additionally, in other studies it was demonstrated that nanomaterials could enhance drug percutaneous penetration [37,38]. However, the mechanism of action of nanoparticles as skin penetration enhancer still has been studying by different research groups. Cai et al. used atomic force microscopy to understand if drug-nanoparticle surface interactions, which appeared during topical application, are able to enhance percutaneous penetration [39]. Tetracaine was used as a model drug. In order to check the drug-nanoparticle interactions two different nanoparticle surfaces (silica nanoparticles and negatively charged carboxyl-modified polystyrene nanoparticles) were applied. The active compound-nanoparticle adsorption was studied in an aqueous vehicle set at pH 4 and pH 8. In force adhesion AFM measurements methyl and ionized tertiary amine tips were applied to represent two ends of a charged tetracaine molecule. At both pH 4 and pH 8 the methyl tip exhibited a preference in terms of adhesion force with modified polystyrene nanoparticles. On the other hand a much lower force of adhesion with methyl tip was observed for silica nanoparticles at both pH conditions. Similar trend was noticed between amine tip and both nanoparticle surfaces.

The reduction in adhesion was connected with the lower surface charge of silica nanoparticles (ca. -23 mV) in comparison with modified polystyrene nanoparticles (ca.-40 mV) that reduced the electrostatic interactions between the negative particle and positive amine of drug [39]. The force adhesion measurements enabled to show that tetracaine strongly adsorbed to the surface of modified polystyrene nanoparticles and therefore the drug permeation could be retarded. The results obtained in this study demonstrated that addition of nanoparticles to a topical formulation might modify the drug percutaneous penetration. However, there should be a proper balance of interaction strength between drug and the surface of nanoparticle. When the interaction is too strong the drug delivery can be retarded. On the other hand when the interaction is moderate the physical characteristics of drug can be modified and thus the transmembrane transport can be enhanced.

Recently, a novel carrier system called nanomiemgel (NMG) consisting of a mixture of nanomicelles (NMI) and nanoemulsion (NEM) for the topical application of aceclofenac (ACE) and capsaicin (CAP) has been discovered. Moreover, the in vitro permeation of the drug through dermatomed human skin and inflamed mice skin was studied by AFM [40].

Additionally, the investigation of synthetic membranes that permits determination of their structure and characterization of their properties was made. Eight synthetic membranes were characterized by using AFM in order to understand their behaviour in the active substance release experiments. The results proved that morphology played an important role, e.g. polytetrafluoroethylene membrane was not suitable for the release study of tetrapeptide due to its hydrophobic nature, thickness and the specific structure with high trapezoid shaped blocks. The additional sub-structures in pores of mixed cellulose esters- and nylon-type membranes showed an influence on the diffusion rate of the active compound. Thus the proper selection of the membrane for the active substance release studies has to be carefully inspected prior to the experiment [41].

4.4 Application of AFM in cosmetology

The characterization of morphological and adhesive properties of skin and hair are of great interest to cosmetic science. The knowledge about tribological properties of hair and skin could be helpful to develop better beauty care products. So far AFM has been used in cosmetology to analyze hair [16, 31, 42, 43, 44, 45]. Atomic force microscopy was applied to investigate nanotribological properties of hair and skin and also the influence of hair care products [46,47] In order to compare changes occurring due to damaging process the roughness parameters and nanoscale friction force were determined. The distribution of conditioner applied on the hair was presented by adhesive force mapping. LaToree et al. performed the nanotribological research of different types of hair and skin as a function of ethnicity, damage and conditioning treatment [46]. The results showed that the adhesive force decreased for damaged hair. However, much higher adhesive force was detected for treated hair that could be related to the accumulation of conditioner on the hair surface. LaTorre et al. suggested that force-volume maps might be a promising way to analyze hair before and after the treatment.

The way in which creams influence the tribological and mechanical properties of skin is of great interest to both pharmaceutical and cosmetic sciences. Therefore, recently AFM have found also application in analyzing the effect of creams on skin surface. Tang et al. carried out tribological studies of virgin skin and skin treated with cream by atomic force microscopy [18]. The AFM was used to study the morphology of skin surface. Additionally, the effect of cream film thickness, normal load, relative humidity, velocity and temperature on the coefficient of friction and adhesive force of treated with cream and non-treated skin was investigated. The cream film thickness was determined by "force distance curve" technique [48,49] in which the tip was brought into contact with the surface of the sample by extending the piezo vertically. Afterwards the piezo was retracted and the force needed to separate the tip from the sample was calculated. The obtained results demonstrated that skin cream reduced the surface roughness and increased the skin hydrophilic properties. When the cream film thickness increases the adhesive force and the coefficient of friction also increased. The studies proved that skin cream could smoothen the surface of the skin and improved its hydrophilic properties. Additionally, this research demonstrated that the adhesive force and coefficient of friction of treated and non-treated skin depended on the cream film thickness, normal load, velocity, relative humidity and temperature.

Similar results were obtained by Bhushan, who presented an overview of nanotribological and nanomechanical properties of skin treated and non-treated with cream [50]. The effect of cream film thickness, velocity, normal load, relative humidity on the adhesive force and coefficient of friction of skin was tested. The results proved that the cream reduced the skin surface roughness and improved skin hydrophilicity. Large load carrying capacity was observed for cream film that suggested that cream could serve as a protection of skin surface. The findings of both studies could be crucial in development of new skin care products.

Atomic force microscopy was also used to study structural changes in stratum corneum after application of terpenes [51]. First the formulations containing terpenes were applied on the forearms. Next tape strips were pressed on the skin to collect the stratum corneum layer. Afterwards the isolated corneocytes were analyzed by atomic force microscopy. 3D topography of corneocytes and surface roughness were examined. For the treated and untreated samples no significant changes in stratum corneum and individual corneocytes were observed.

Starostina et al. demonstrated that AFM could be applied in the cosmetic industry to develop the advanced skin care products [52]. The 3D images of the skin surface replica before and after treatment were analyzed. It was shown that the depth and surface roughness were greater for the treated samples. It suggests that the ant-wrinkle activity of topical anti-wrinkle formulation could be successful. The quantitative assessment of anti-aging products in nonoscale could be assessed. The data obtained proved that AFM might be used to analyze the changes occurring in the skin

Gaikwad et al. developed a protocol to determine the changes in corneocytes properties after the treatment with a moisturizer such as Olay Quench body cream [21]. Five volunteers participated in this study. The skin flakes were collected from the forearm by adhesive tape strips before and after the treatment. Smoothening of the corneocyte surface, an increase of friction, and a decrease of rigidity were observed after cream treatment. AFM enabled to detect early changes in corneocytes. Gaikwad et al. suggested that AFM could be applied as a screening technique for development of the topical formulations. Dulinska-Molak et al. performed similar studies. AFM was applied to check the influence of a moisturising cream on skin [10]. Changes in the rigidity of corneocytes were determined by assessing the Young's modulus values which were estimated by Johnson-Kendall-Roberts model [53, 54]. The JKR model takes into account the existence of adhesion forces. This model is used to study the rigidity of cells of stratum corneum because it assumes the presence of a linear strain-deformation ratio and lack of long-distance interactions [10]. The results proved that corneocytes, which were not treated with cream, had different mechanical properties and are more rigid than corneocytes treated with cosmetic. Additionally, corneocytes were smoother and their adhesion force increased after treatment with moisturizing cream. Based on these results authors suggested that AFM could be applied as a new tool in the development of new skin care products [10].

It is well know that psoriasis affects not only the "soft" skin keratin, but also "hard" keratin, such as hair. The changes in the hair of patients, which had psoriasis were monitored by using AFM. Such study performed by Shin et al. proved that changes in hair structure were analogous to the changes in skin and nails [55]. The same authors investigated the differences on the hair surface in scalp psoriasis (SP) and seborrheic dermatitis (SD) using AFM [56].

4.5 Development of skin equivalents

Synthetic skin could be used for wound healing and also in pharmaceutical and cosmetic industries. Skin equivalents are required for toxicity and permeation studies [57]. The synthetic skin should have similar properties to natural skin. Therefore, it is important to know how to assess the mechanical and tribological characteristics of the skin substitutes.

Chen and Bhushan performed studies on nanomechanical and nanotribological properties of two synthetic types of skin before and after treatment with cream [58]. The hardness, surface roughness, elastic modulus, adhesive force and film thickness of synthetic skins were measured by AFM. The results were compared with that of rat and pig skin because in cosmetic science it is important to obtain a skin equivalent with similar properties to that of animal skins. The properties of two synthetic skins were comparable to the animal skin. The results showed that skin cream had an influence on the properties of skin surface. The cream film increased the surface film thickness that led to increase in the adhesive and friction forces. Additionally, the cream reduced the skin surface roughness. Furthermore, hydrophilic properties of skin increased due to the application of the cream.

5. Conclusions

The review demonstrates the basic concepts of atomic force microscopy and the recent progress in relation to skin research. The increasing number of published papers indicates that this method could be a powerful tool for analysis of skin in nanoscale. AFM enables to get the morphology of skin in a good resolution. Additionally, it gives possibility to obtain not only the topographical image of skin but also quantitative data about its mechanical properties. Therefore this could be considered as a main advantage of AFM over other imaging analysis. Furthermore, atomic force microscopy could be used to determine the impact of topical formulations on skin. However, since skin properties range widely for different individuals it is still much to study to understand the mechanism of drug delivery though skin or the impact of different skincare products.

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