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ATR-IR study of skin components: Lipids, proteins and water. Part I: Temperature effect

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ABSTRACT

In this work we report the studies of the effect of temperature on skin components, such as lipids, proteins and water. Modifications of lipids structure induced by increasing temperature (from 20 to 90 °C) have been studied using ATR-IR (Attenuated Total Reflectance Infrared) spectroscopy, which is a powerful tool for characterization of the molecular structure and properties of tissues, such as skin. Due to the small depth of penetration (0.6–5.6 μm), ATR-IR spectroscopy probes only the outermost layer of the skin, i.e. the *stratum corneum* (SC). The assignment of main spectral features of skin components allows for the determination of phase transitions from the temperature dependencies of band intensities [e.g. $\nu_{as}(CH_2)$ and $\nu_s(CH_2)$]. The phase transitions were determined by using two methods: the first one was based on the first derivative of the Boltzmann function and the second one employed tangent lines of sigmoidal, aforementioned dependencies. The phase transitions in lipids were correlated with modifications of the structure of water and proteins.

1. Introduction

Skin is a barrier that protects the body against external mechanical, physical, chemical and biological factors. From the histological point of view, it is a highly multilayer composite consisting of three main structures: epidermis, dermis, and hypodermis. The epidermis is the outermost part of the skin directly separating the body from the environment, with thickness ranging from 50 to 150 μm. It consists of five layers composed of a tightly adjacent, maturing epithelial cells, keratinocytes: *stratum basale*, *stratum spinosum*, *stratum granulosum*, *stratum lucidum* and *stratum corneum*, the latter one being the outermost skin layer [1]. In the process of keratinization (keratosis) keratinocytes gradually transform to build these layers, each having specific properties. Epithelial cells moves towards skin surface changing their morphology mainly due to increasing content of keratin, accompanied by secretion of lipids. At the end of this process cells dehydrate and die, resulting in the formation of flat cells without nuclei and filled with the insoluble protein keratin called corneocytes, 0.2–0.3 μm thick and 30–50 μm in diameter [2]. Corneocytes bonded together with lipid matrix, form the most outside epidermal layer called *stratum corneum* (SC), which serves primarily as a protection against physical stimuli and chemical agents for lower lying layers of the skin and also prevents from water loss.

Stratum corneum is only 10–20 μm thick and its structure is often called "*bricks and mortar*", where the corneocytes represents the bricks and lipids are the mortar between them. The corneocytes consists primarily of soft keratin (about 95%), minor amounts of other proteins and water. The lipid matrix contains ceramides (45–50% by weight), cholesterol (20–25%), free fatty acids (10–15%), and derivatives of cholesterol and glucosylceramides (15%) and also catabolic enzymes that cause the transformation of the polar lipids into the non-polar compounds, insoluble in water [3–7]. Correct composition and quantity of lipids in the epidermis is necessary for maintaining the integrity between the cells and formation of a protective barrier on the skin surface [8].

One of the most common factors causing damage of the skin is temperature, which affects, structure of lipids, proteins and water contained in the tissue. The effect of temperature on its biological condition may be positive, what is used e.g. in thermotherapy, where skin is exposed to temperatures falling out of the optimum range, i.e. between 20 °C and 30 °C. For example enlarged temperature causes increase of the permeability of the skin due to lowered density of lipid matrix in stratum corneum, what may be used, inter alia, in transdermal application of drugs [9].

High temperature causes irreversible changes in the structure of skin proteins in the process called denaturation. Characteristic bonds break, polypeptide chains lose their specific spatial organization, unfold and adopt very often a random conformation. For example the ordered structure of triple-helical collagen, which is formed by three connected polypeptide chains, is unfolding to form the random coil structure [10].

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The denaturation is accompanied by loss of biological properties of proteins, e.g. loss of enzymatic activity.

Numerous biophysical methods have been used to study skin components and the relation between the structure and functions at the molecular level, including: DSC (differential scanning calorimetry) [11,12], X-ray diffraction [13–15], neutron diffraction [16], electron microscopy (TEM, SEM, or Cryo-SEM) [14,17], NMR (nuclear magnetic resonance) [18–20], EPR (electron paramagnetic resonance) [21,22], OCT (optical coherence tomography) [23,24] vibrational {NIR, FTIR, ATR-IR [11,12,14,25–28], Raman (FT, NIR, and confocal) [28–33]}, AFM-IR [34,35] and NIR diffuse reflectance spectroscopy [36] or dynamic vapour sorption (DVS) [29]. Particular attention should be paid to FTIR. This spectroscopy is a non-invasive tool that permits for rapid characterization of tissue by detecting changes, even very subtle, at the molecular level. Vibrational spectroscopy is very helpful to identify the tissue, their components, and to differentiate between healthy and pathological samples, according to the changes in structure and chemical composition of tissue components.

The ability to identify the biochemical composition of the skin makes infrared spectroscopy a method allowing for diagnose of cancerous changes at the very early stages of the disease. Spectroscopic methods can quickly detect pathological changes in the structure of the proteins, may be helpful in studying the aetiology of skin diseases and evaluation of their progress by imaging the conformational changes of lipids, proteins and other components of the skin [37].

FT-Raman [38,39] and IR spectroscopy [40,41] allow to determine modifications of molecular structure of biological tissues such as skin. Molecules or molecular fragments that cannot be accurately detected with one method can be easily detected with the other technique. Therefore, researchers use them simultaneously or in a complementary way. Most of the experimental techniques used by vibrational spectroscopy are non-destructive, and allow to analyze samples in various states, both in vitro and in vivo by using the fiber optics. Raman spectroscopy offers advantages over FTIR one because Raman spectra are not affected by the interference of water signals. In contrast, strong absorption from water in IR region creates serious problems in interpretation of the spectra, especially for hydrated samples. NIR spectroscopy can also be used in analysis of aqueous solutions. For IR and Raman measurements a small amount of sample is sufficient (for example, samples from biopsy), however, NIR spectroscopy is the best approach for recording of the spectra of the whole samples (i.e. whole human head). NIR spectroscopy offers deep sample penetration, high sensitivity and signal energy, being significant method in studies of skin tissue. Besides, NIR bands are significantly weaker than MIR bands and, therefore more convenient film thicknesses can be used. NIR region is dominated mainly by absorption from functional groups with a hydrogen atom (OH, NH, CH). Band shift due to molecular interactions and hydrogen bonding is larger in NIR than that in MIR region. As a result, the bands due to different associates are better separated in the NIR spectra. The intensities of polar groups are relatively high in MIR spectra. On the other hand, hydrophobic and aromatic groups, multiple and symmetric bonds, are better visible in Raman spectra. The Raman spectra are usually recorded in glass or quartz cells, thus avoiding the inconvenience of working with sodium chloride (NaCl) or other atmospherically sensitive window materials. However, Raman spectroscopy requires high-stability laser sources and efficient amplification equipment to detect the weak signals. This makes Raman instruments more expensive compared to IR ones.

Additionally, various quantum chemical approaches can be applied for simulation of IR spectra to help interpretation of absorption intensities and wavenumbers of the fundamental, overtones and combination vibrations and their anharmonicities [42,43]. In the case of whole tissue it is very difficult to perform such calculations. However, in the literature one can find molecular dynamics study of *stratum corneum* of lipid mixtures [44].

The scope of this work includes: a) determination of the lipid phase transitions in the temperature range of 20–90 °C, b) study of the temperature-induced conformational changes, c) monitoring the changes in the composition of the skin. To our best knowledge, for the first time the effect of temperature on various components of the skin was studied simultaneously. Structural changes in skin components (lipids, proteins and water), including those induced by temperature, have important consequences for the functional properties of skin and this study advances the understanding of the reactivity of biological molecules and mechanisms of thermal processes at the molecular level. ATR-IR spectroscopy was selected as a very powerful tool to study various components of biological tissues [45–48]. Due to its high sensitivity for structural changes of tissue components, and inter- and intramolecular interactions, this method has been recognized as a promising tool for rapid and highly-informative molecular characterization of normal and pathological tissues in vitro, ex vivo and in vivo [49–51]. For example, ATR-IR spectroscopy has been applied as a versatile tool to study human SC barrier function in vivo [26], lipid organization or composition [26,27], water content of the SC or skin hydration [26,52,53], dermal drug delivery [49] or even skin cancers [49,50,54].

2. Materials and Methods

2.1. Reagents

Solid NaCl of purity not <99% was obtained from POCH S.A (Gliwice, Poland), and glycerine was purchased from Stanlab (Lublin, Poland). Both reagents were used as received without further purification.

2.2. Treatments

Aqueous solutions of NaCl were prepared with deionised water at a physiological concentration of 0.9%. Next, glycerine was dissolved in NaCl solution (5:1 ratio) in order to receive 20 wt% cryoprotectant solution.

2.3. Skin Preparation

Healthy skin, which was used for the study, is of porcine origin (Polish Landrace breed, age of the pigs: two months). Skin was provided by Department of Surgery the Faculty of Veterinary Medicine, Wrocław University of Environmental and Life Sciences, Wrocław, Poland. The skin was taken from pigs with the approval of the Second Local Ethics Committee for Affairs of Experiments on animals. After removal of excess hair and the subcutaneous fat, the skin was stored in the presence of cryoprotectant (20 wt% glycerine) solution.

2.4. Thawing and Washing Procedure

Prior to collection of ATR-IR spectra, the procedure of thawing was carried out very slowly and gradually, thus as not to damage the studied tissue. The next step was to wash the material in a 0.9% NaCl solution to remove the glycerol. Skin sample washing time was determined experimentally, i.e. washing has been continued till complete disappearing of glycerol bands from IR spectrum of a sample.

2.5. Measurements

All the samples were prepared from the pig skin. FTIR spectra were measured in vitro in the range of 700–4000 cm^{-1} on a NICOLET 6700 spectrometer (Thermo Scientific, USA) with Golden Gate Mk II ATR Accessory with Heated Diamond Top-plate (PIKE Technologies) [55]. Samples were placed directly on the diamond crystal and covered to eliminate the evaporation of water. Spectra were recorded at constant temperature.