Effect of Plasma on Structure and Permeability of Epidermal Layer of Pig Skin

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Abstract— Transdermal drug delivery requires changes of properties of the skin to increase its permeability. Effect of plasma jet treatment of Stratum corneum was investigated. Histological observation of the cross section of the skin characterized etching effect of plasma depending on the treatment time. Changes of protein and lipid conformations were observed by ATR-FTIR spectroscopy. Plasma jet caused structural changes observed by increase of bandwidth and decrease of absorbance of the lipid vibrational bands. These changes can increase flexibility of lipid structures and permeability of the skin.

I. Introduction

Epidermal layer of the pig skin is composed of viable epidermis and stratum corneum. Stratum corneum is formed by death cells placed in a lipid matrix. They are main barrier of the body which prevents foreign substances to enter the body. However, this barrier function is undesirable, when the skin is used as a route for a drug delivery. In such situation, the skin and mainly stratum corneum must be disturbed. Drugs with molecular weight less than 500 Da are the ideal candidates for the transdermal delivery because they can pass through the skin very often without any difficulties. Nevertheless, penetration enhancers are necessary for achievement of the therapeutic amount in blood [1]. Relatively heavy drugs such as Cyclosporine A which molecular weight of 1203 Da is impossible to deliver to the body without any enhancer. Plasma jets are known in field of healing of injuries or wrinkle treatments [2], but not so much as skin permeability enhancers what is possible to see in number of the published papers [3-7]. Argon plasma was used for the skin treatment and it can be considered to enhance permeability of the skin what was proved by [7]. As the most populated species are argon atoms, argon ions and argon metastable states with impurities coming from atmospheric air. Bombardment of the skin surface by ions and followed by reaction with released oxygen, nitrogen or hydrogen can lead to the etching effect of the skin surface. Additionally, the histological observation of the elimination of the corneocytes verified etching effect of the plasma where thickness of stratum corneum was significantly decreased. Plasma can change structure or chemical composition of the skin to enhance their permeability. Changes caused by plasma were

observed by using Attenuated Total Reflectance – Fourier Transform Infrared (ATR-FTIR) spectroscopy. The measurement of the slight shift or vibrational band broadening of the symmetric and asymmetric stretch of methylene peaks demonstrated the plasma-induced disruption of the lipid bilayer. Changes in C=O vibrations in ATR-FTIR spectra induced chemical reactions with molecules in the stratum corneum. Plasma effect on protein structure was also observed and detected by deconvolution of Amide I band belonging to keratin.

II. Experiment

A. Experimental setup

Plasma is generated by a plasma jet described before in ref. [8]. Atmospheric argon plasma was maintained by a voltage of 4 kV and a frequency of 16 kHz by a Neon transformer (ALPHA Neon M-5). Flow of argon gas (purity 99.999%) was set to 3 L/min by a flow meter (Yamato). The distance between the skin sample and the outlet of the plasma jet was set to 2 mm via a grounded micrometric sample holder. The sample was isolated from the holder by a 30-mm thick PVC isolator. The treatment time of the sample was set from 10 s to 900 s (15 minutes). Attenuated Total Reflectance-Fourier Transform InfraRed (ATR-FTIR) spectrometer (Jasco FT/IR 6300 with ATR PRO610P-S) with a diamond prism was used to observe the upper layer of stratum corneum of pig skin. Spectra were recorded with a resolution of 8 cm^{-1} and by accumulating 150 scans.

B. Sample preparation

Pig skin of Yucatan micropig from Charles River Japan, Inc. (Yokohama, Japan) was used for investigating of the influence of the plasma treatment. The pig skins were stored at −20 °C in a freezer before the experiment. The fat layer of the skin was removed, then it was cut and soaked at 4 °C in phosphate buffered saline (PBS) for 3 h, and then the epidermal layer was removed after a bath in 60 °C PBS for 1 min. Finally, the skin sample was cut to 3×3 mm pieces and attached to polypropylene film by using double-sided tape.

III. Histological observation of the skin section

A small piece (approximately 15 × 15 mm^2) of the treated skin was cut and fixed in 10% neutral buffered formalin for four days. The formalin solution contains stock of formaldehyde solution (35% CH_2O, 1 L), sodium phosphate monobasic ($NaH_2PO_4 \cdot H_2O$: 44 g), sodium phosphate dibasic anhydrous (Na_2HPO_4, 65 g) and distilled water (9 L). After four days, the skin was dehydrated and embedded into paraffin mold. The paraffin-embedded skin sample was sectioned at a thickness of several micrometers using a microtome and placed on a glass side. In order to visualize the micro-scale change in the stratum corneum, hematoxylin-eosin staining was then performed. The layer of stratum corneum in Fig. 1A has thickness close to 30 μm. After 10 s treatment by plasma jet, thickness decreased to 20 μm, 18 μm after 30 s of treatment and 10 μm after 60s of treatment. However, as it is seen in Fig. 1B, some spots on the skin are disrupted more and thickness is close to 3 μm.

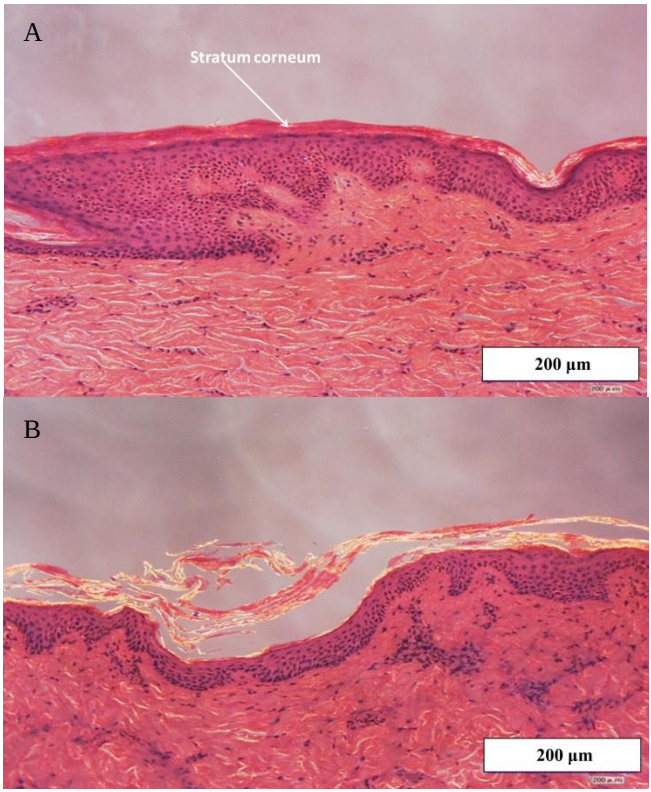

Fig. 1. Histological cross sections of the skin A:nontreated skin B: skin treated by plasma jet for 60 s.

IV. ATR-FTIR ANALYSIS

There are several methods of analyzing skin structure and composition such as Raman spectroscopy [9], x-ray diffraction [10], electron diffraction [11] and transmission electron microscopy [12]. ATR-FTIR can tell us information about skin hydration, protein and lipid structure [13, 14].

A. Water OH bands

Fig. 2 shows typical ATR-FTIR spectra of the stratum corneum layer (white line). Spectrum is overlapped by many vibrational bands and analysis is not simple. A huge part of the spectrum is covered by OH bands coming from liquid water present in the epidermal layer (green line in Fig. 2). To subtract these bands, spectrum of distilled water was measured. This spectrum was scaled so that after substraction, zero spectrum was achieved in the range (1800 – 2000 cm^{-1} and 3400 – 4000 cm^{-1}). Resulted spectrum (blue line in Fig. 2) was analyzed further. According scaling factor, we were able to detect relative changes of water content in stratum corneum. Plasma jet treatment caused decrease of water content in stratum corneum to approximately 30% of initial value. This value was saturated between 120 and 360 seconds of treatment (Fig. 3). There are two reasons of increased water

evaporation after plasma treatment of the skin. The first one is heating the sample. The surface temperature of the skin sample was 60°C after 60 s of irradiation by plasma jet [7]. The second one is increase of permeability of the stratum corneum [3].

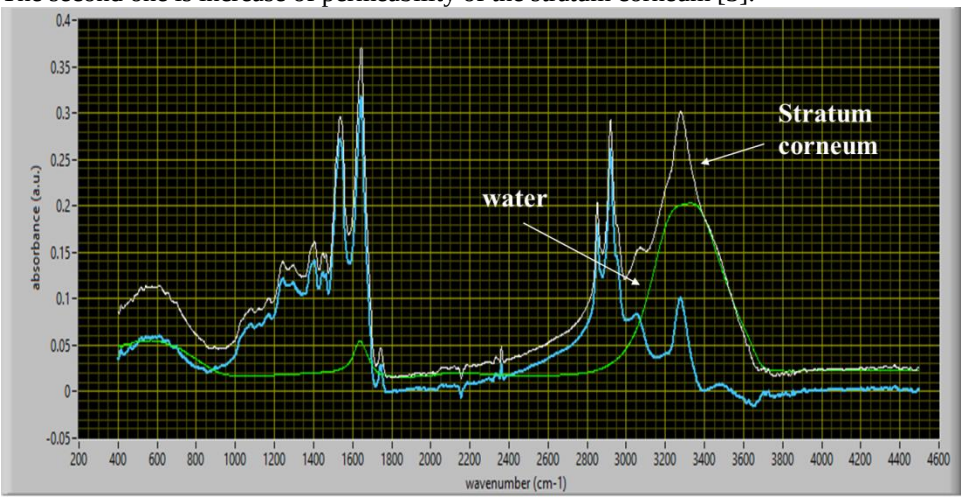

Fig. 2. Spectrum of stratum corneum (white), spectrum of water (green), stratum corneum spectrum after substraction of water.

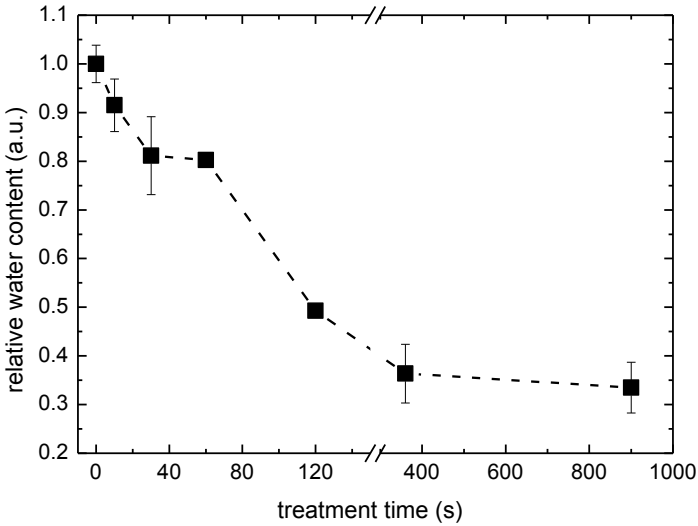

Fig. 3. Relative water content in stratum corneum during plasma treatment.

B. Methyl CH_3 and Methylene CH_2 bands

Bandwidth and absorbance determined by area of the vibrational bands depicted by Fig. 4 were analyzed. Lipids of the stratum corneum are characterized by methylene symmetric and asymmetric stretches at 2850 cm^{-1} and 2920 cm^{-1} and methyl asymmetric stretches at

2955 cm^{-1} (Fig. 4A). Methyl symmetric stretches vibration comes from proteins. These bands were analyzed by Gaussian fit. To increase precision of the fit, also 2nd derivation of the spectra was fitted. Second derivation was scaled to have approximately the same effect on result as spectrum (Fig. 4B). Asymmetric stretching band of CH_2 was simulated by three Gaussian peaks (Fig. 4A).

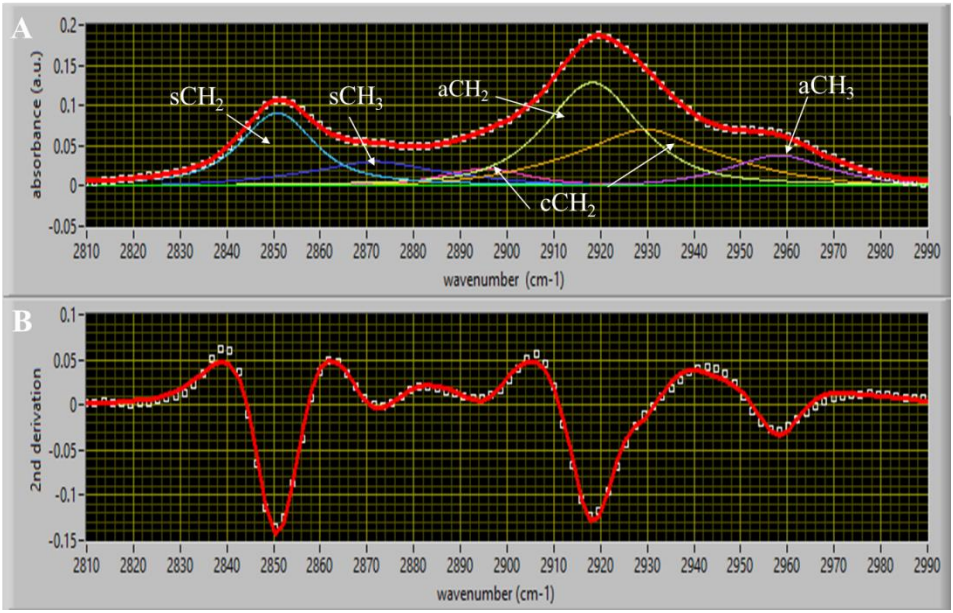

Fig. 4. Simulation of methyl and methylene symmetric and asymmetric stretches of CH_3 and CH_2.
A: Experimental spectrum (white points) with simulated spectrum (red). Resolved peaks are sCH_2 (symmetric stretches vibration of CH_2), aCH_2 (asymmetric stretches vibration of CH_2), sCH_3 (symmetric stretches vibration of CH_2), aCH_3 (symmetric stretches vibration of CH_3), two components of cCH_2 (two components of the asymmetric stretches vibration of CH_2),
B: The second derivation of experimental spectrum (white points) and the second derivation of simulated spectrum (red).

Absorbance of all bands decreased what induced extraction of lipids as it is shown in Fig. 5. We could also observe change of bandwidth what is connected with structural changes of lipids. Bandwidth of asymmetric methylene stretching band increased from 16 cm^{-1} to 19 cm^{-1} and symmetric methylene stretching band increased 14.7 cm^{-1} to 16.5cm^{-1}, as it is shown in Fig. 6. In the case of the bandwidth and also absorbance, saturation effect was achieved after 120 s of treatment of the skin.

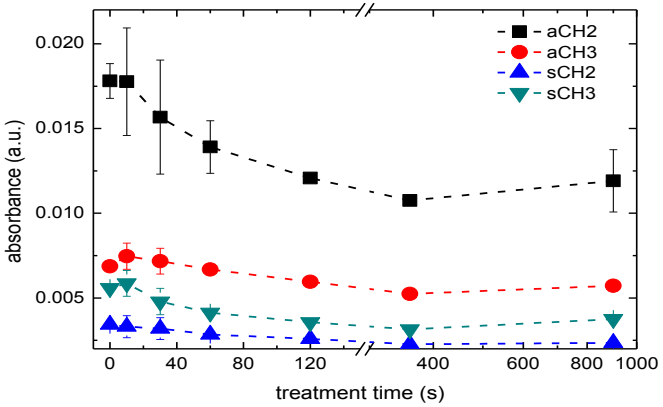

Fig.5. Absorbance of methyl and methylene symmetric and asymmetric stretches of CH_3 and CH_2. sCH_2 is the symmetric stretches vibration of CH_2, aCH_2 is the asymmetric stretches vibration of CH_2, sCH_3 is the symmetric stretches vibration of CH_2, aCH_3 is the symmetric stretches vibration of CH_3.

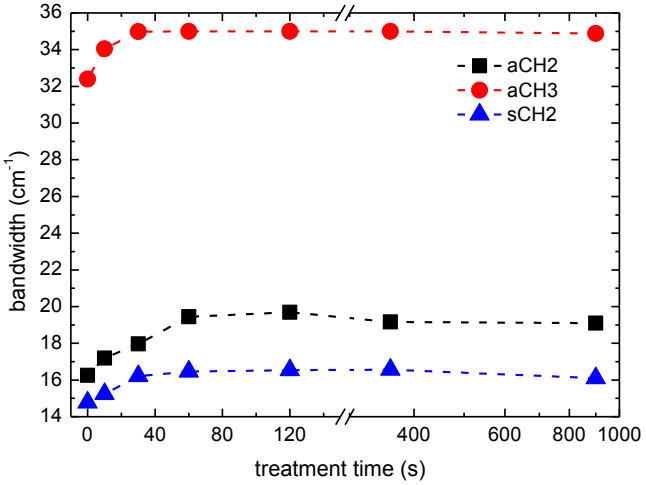

Fig. 6. Bandwidth of methyl and methylene symmetric and asymmetric stretches of CH_3 and CH_2. sCH_2 is the symmetric stretches vibration of CH_2, aCH_2 is the asymmetric stretches vibration of CH_2, sCH_3 is the symmetric stretches vibration of CH_2, aCH_3 is the symmetric stretches vibration of CH_3.

C. Amide A band

Amide A band comes from the N-H stretching of proteins. This band is insensitive to protein conformations and exclusively depends on N-H bond what allows us to determine protein concentration from absorbance of the band [15]. Position of Amide A depends on strength of hydrogen bond [16]. According Olsztynska-Janus *et* al., when N-H is involved in hydrogen bond, N-H stretching vibration can be shifted to lower wavenumbers. Weidner *et* al. showed that position of N-H stretch depends on side chains. Generally, hydrogen

bond decrease wavenumber of stretching vibrations and increase wavenumber of bending vibrations [16]. We observed Amide A peak at 3280 cm^{-1} and after 120 seconds of plasma jet treatment was shifted towards lower wavenumbers by average 2.5 cm^{-1} (Fig. 7).

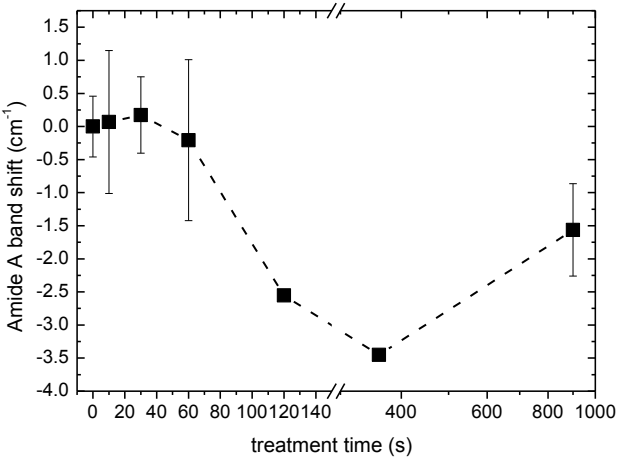

Fig. 7. Amide A band shift during the plasma treatment.

D. C=O stretching vibrational bands

Position of C=O stretching band strongly depends on structure of molecule and it can be found from 1600 cm-1 to 1780 cm^{-1}. C=O stretching bond at 1744 cm^{-1} belong to ester bonds [19] and also skin sebum. Sebum is composed from glycerides, free fatty acids, wax esters, squalene, cholesterol esters and cholesterol [20]. These sebum molecules were partially exctracted/decomposed by plasma treatment (Fig. 8).

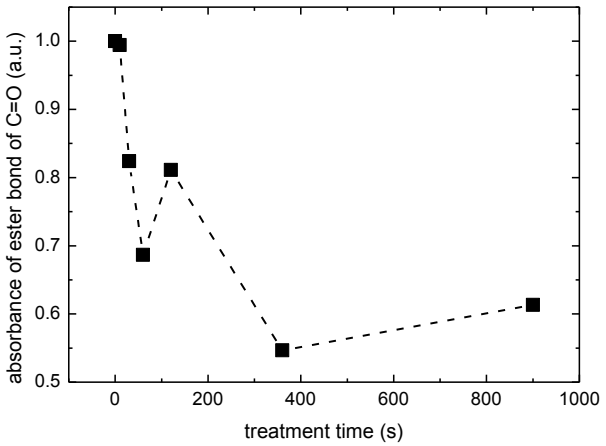

Fig. 8. Absorbance of ester C=O stretching vibration of ester bond during plasma treatment.

C=O stretching bands from amide are located from 1600 to 1700 cm^{-1} and they strongly depend on structure of proteins. Position of peaks of Amide I band was determined by the second derivation of the experimental spectra. Resolved spectrum of Amide I is in Fig. 9. Plasma treatment of skin caused increase bandwidth of alpha-helix and 3_{10}-helix structure by 1 cm^{-1}. Other structures were not changed. Amide I was composed by 44.5% of helix structure, 32% Beta-sheet structure, 9.4% Beta-turn structure and 14% of side chains.

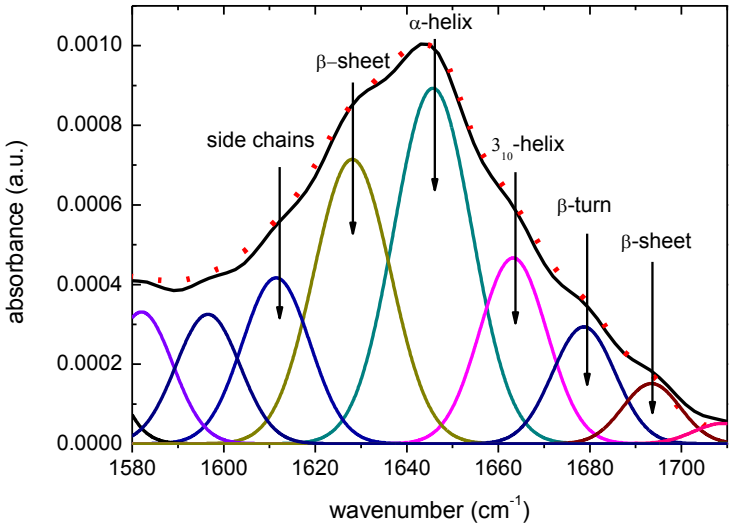

Fig. 9. Simulation of C=O stretches vibrations of Amide I by Gaussian of alpha-helix and 3_{10} helix structure, Beta-sheet structure, Beta-turn structure and of side chains.

V. Conclusion

Plasma is investigated in the field of skin care or drug delivery and knowledge of an effect on the skin is desired. Plasma jet treatment of stratum corneum was realized from 10 s up to 900 s. All main changes in the skin were done up to 120 s and after this time, saturation process became. Water level of the skin reached 30% of initial state. Decrease of absorbance of lipid vibrations in wavenumbers range 2800 – 3000 cm^{-1} and also sebum peak at 1744 cm^{-1} induced lipid extraction. Increase bandwidth has origin in change of structure of lipids which became more flexible. Slight shift of Amide A band meant weaker N-H bond in amide structure of keratin. Amide I structures remained stable except alpha- and 3_{10}-helix that increased bandwidth about 1 cm^{-1} and increased flexibility of the helical structure. The increase of flexibility of lipids and protein helical structures increases the permeability of the skin and helps to drugs the transdermal delivery.

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