Effect of Plasma Discharge on Epidermal Layer Structure in Pig Skin

Ahmad Guji Yahaya,^{a,*} Jaroslav Kristof,^b Marius Blajan,^b Fariha Mustafa,^b & Kazuo Shimizu^{a,b,c}

^aGraduate School of Science and Technology, Shizuoka University, Japan; ^bGraduate School of Integrated Science and Technology, Shizuoka University, Japan; ^cOrganization for Innovation and Social Collaboration, Shizuoka University Japan

*Address all correspondence to: Ahmad Guji Yahaya, Graduate School of Science and Technology, Shizuoka University, Japan; Tel./Fax: +81-53-478-1443, E-mail: Yahaya.ahmad.guji.19@shizuoka.ac.jp

ABSTRACT: Plasma can efficiently inactivate microbial pathogens. Application of plasma on the skin can not only kill bacteria but also change the nature of skin. The influence of plasma jet treatment on the stratum corneum was investigated using the skin of a Yucatan micro pig. Microscopic observation of the histological skin section was used to characterize the etching effect of plasma jet depending on treatment time. Changes in lipid conformations were determined by the bandwidth and absorbance of the symmetric and asymmetric stretching of CH₂ and CH₃ bands and their ratios using ATR-FTIR spectroscopy. Plasma jet caused structural changes observed via an increase in bandwidth and a decrease in absorbance of the lipid vibrational bands. Plasma effects were saturated after 2 min of treatment. The influence of temperature and UV irradiation was also evaluated. The monitoring of plasma-treated skin demonstrated the temporal effect of changes caused by plasma.

KEY WORDS: FTIR, lipids, plasma jet, stratum corneum, skin treatment

I. INTRODUCTION

Plasma generates highly reactive species (ions, electrons, excited atoms, molecules, radicals, metastable states, UV radiation, and heat).¹ Some of these species, particularly reactive nitrogen species (RNS), penetrate and dissolve in some tissues up to 1.25 mm thick.² If not carefully controlled, these species cause tissue damage^{3,4} in biomedical applications of plasma⁵ such as wound healing, skin treatments,^{6–9} transdermal drug delivery for pig and human skin,^{10–17} or sterilization.^{18,19} Except for sterilization of medical devices, it is also necessary to decontaminate human tissue such as hands or body parts prior to surgery or injection. Hand sanitizing by plasma and the plasma jet device has been investigated.^{20–22} However, application of plasma to the skin can not only kill bacteria but also cause some changes to the skin itself. Thus before application, it is crucial to evaluate possible effects of repetitive use because plasma can decrease skin thickness and also decrease the skin's barrier properties.

The epidermal layer of the skin is composed of a viable epidermis and the stratum corneum.²³ The stratum corneum is formed by dead cells in a lipid matrix. Dead cells and lipids are the body's main barrier against foreign substances.^{24,25} They also exhibit a selective permeability and allow only relatively lipophilic compounds to diffuse into

the lower layers.²⁶ Choi et al.²⁷ reported that plasma reduced the skin's barrier properties through reduction of E-cadherin expression; they were revived 3 h after plasma treatment. E-cadherin is a cell adhesion molecule that is very important in the formation of junctions to bind cells to each other.²⁸ Lee et al.²⁹ compared argon and helium discharge; they found argon discharge to be more effective than helium discharge in E-cadherin regulation (decrease in the barrier function) because of higher density of reactive species. They also observed that ions played a key role in enhancing skin permeability. Argon discharge contains the most populated species: argon atoms, argon ions, and argon metastable states with impurities coming from atmospheric air.

Depending on the purpose and setup of the plasma application, etching on the skin surface and the grafting of lipids by air particles are possible results of bombardment by ions, followed by reaction with released oxygen, nitrogen, or hydrogen. Plasma can change the structure or chemical composition of the skin to enhance permeability.³⁰ In this study, changes caused by plasma were observed using attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectroscopy. Effects of treatment time, temperature, and radiation were evaluated along with posttreatment behavior. Structural changes in lipids was measured according to bandwidth and absorbance of symmetric and asymmetric stretching of CH_2 and CH_3 bands and their ratios. Histological observation of skin sections determined the skin-etching effect.

II. EXPERIMENT

This research focused only on the effect of plasma jet irradiation on the stratum corneum. Previously published papers^{31–33} confirmed that cold atmospheric pressure plasmas can cause internal skin changes. To characterize these effects, microscopic observation of histological skin sections was used to observe the etching effect of plasma jet depending on treatment time. Changes in lipid conformations were also observed via bandwidth, as were the absorbance of the symmetric and asymmetric stretching of CH_2 and CH_3 bands and their ratios using FTIR-ATR spectroscopy.

A. Experimental Setup

Plasma jet³³ was generated using 99.99% pure argon gas (Fig. 1). The flow was set at 3 L/min by a flow meter (Yamato). The discharge voltage was ignited at 4 kV and at a frequency of 16 kHz by a neon transformer (Model M-5, Alpha Neon). The distance between the skin samples and the outlet of the plasma jet was set at 2 mm via a grounded micrometric sample holder. The samples were isolated from the holder by a 30-mm-thick PVC isolator. Treatment time was set at 10–900 s (15 min). The effect of UV radiation was evaluated by use of a filter between the plasma jet and the sample. The temperature effect was tested using a hot air fan. The upper layer of the stratum corneum was analyzed using a diamond prism ATR-FTIR spectrometer (FT/IR 6300, Jasco, with PRO610P-S, ATR). The spectra were recorded at a resolution of 8 cm^{-1} and by accumulating 150 scans.

FIG. 1: Experimental setup for plasma jet

B. Sample Preparation for ATR-FTIR Measurements

Yucatan micropig skin, purchased from Charles River Japan was stored at -20°C in a freezer before the ATR-FTIR experiment. After removing its fat layer, the skin was cut and soaked at 4°C for 3 h in phosphate buffered saline (PBS). After a water bath at 60°C for 1 min, the epidermal layer was removed and the sample was cut into 3×3 -mm pieces and attached to a polypropylene film using double-sided tape. Sample thickness was $150\text{--}200\text{ }\mu\text{m}$. The depth of analysis was $1.317\text{ }\mu\text{m}$ at 650 cm^{-1} and $0.214\text{ }\mu\text{m}$ at $4,000\text{ cm}^{-1}$. The FTIR spectrum was corrected to compensate for these differences.

The full-thickness skin samples without the fat layer were cut into smaller pieces (approximately $15 \times 15\text{ mm}$) for microscopic observation (skin sectioning) and divided into two groups. One group was treated with plasma jet; the other was not treated. Afterward treatment, the samples were cut and fixed for four days in 10% neutral buffered formalin containing sodium phosphate monobasic (44-g $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$), formaldehyde solution (1-L 35% CH_2O), sodium phosphate dibasic anhydrous (65-g Na_2HPO_4), and distilled water (9 L). After the four days, the samples were dehydrated and embedded into paraffin molds, sectioned at a thickness of several micrometers using a microtome, and placed on glass slides. To visualize the change in the stratum corneum at microscale, hematoxylin-eosin staining was performed.

III. RESULTS AND DISCUSSION

A. Microscopic Observation

The stratum corneum layer in Fig. 2(a) had an average thickness close to $30\text{ }\mu\text{m}$. After 10 s of plasma jet treatment, the average thickness decreased to about $20\text{ }\mu\text{m}$; after 30 s of treatment, to $18\text{ }\mu\text{m}$; and after 60 s of treatment, $10\text{ }\mu\text{m}$ [Fig. 2(b–d), respectively]. Thickness was determined based on an average thickness measured by microscope at 10 different spots.

FIG. 2: Histological cross sections of (a) nontreated skin sample, (b) 10 s, (c) 30 s, and (d) 60 s

B. ATR-FTIR Analysis

There are several methods of analyzing skin structure and composition. These include Raman spectroscopy,³⁴ X-ray diffraction,³⁵ electron diffraction,³⁶ and transmission electron microscopy.³⁷ ATR-FTIR can tell us about skin hydration and about the structures of lipids or proteins.^{38,39}

1. Water OH Bands

Figure 3 is a typical ATR-FTIR spectrum of the stratum corneum layer (black line). The spectrum is overlapped by many vibrational bands and the analysis is not simple. An extremely large portion of the spectrum is covered by OH bands resulting from the liquid water present in the epidermal layer. To subtract these bands, the spectrum of distilled water was measured. The green or water line in Fig. 3 represents the infrared spectrum of the distilled water. The water spectrum was scaled in a such way that after the subtraction, a zero spectrum was obtained in the ranges 1,800–2,000 cm^{-1} and 3,400–4,000 cm^{-1} . The resulting spectrum (blue or middle line) was analyzed further.

2. Methyl CH_3 and Methylene CH_2 Bands

The bandwidth and absorbance of the vibrational bands depicted in Fig. 4 were analyzed. Lipids of the stratum corneum were characterized by the methylene symmetric

FIG. 3: Spectrum of stratum corneum (black), water (green), and after subtraction of water (blue or middle line)

FIG. 4: Symmetric and asymmetric stretch simulation of methyl and methylene, FTIR spectrum (top) and second derivative (bottom). Dotted lines represent experimental spectrum; red or solid line on top of dotted line represents sum of Gaussian fits.

and asymmetric stretches at 2,850 cm⁻¹ (sCH₂) and 2,920 cm⁻¹ (aCH₂), and the methyl symmetric stretches at 2,865 cm⁻¹ (sCH₃) (Fig. 4). The methyl asymmetric stretch

vibration at $2,955\text{ cm}^{-1}$ (aCH_3) came from proteins. The small band at $2,900\text{ cm}^{-1}$ (fCH_2) was caused by the Fermi resonance of the CH_2 symmetric stretch. The peak centered at $2,930\text{ cm}^{-1}$ (fCH_3) was due to the CH_3 Fermi resonance.⁴⁰ These bands were fitted by a Gaussian function. To increase the precision of the fit, the second derivative of the spectra was also simulated, scaled to have approximately the same effect on the result as the experimental spectrum (Fig. 4). The absorbance of the methylene symmetric and asymmetric stretches at $2,850\text{ cm}^{-1}$ (sCH_2) and $2,920\text{ cm}^{-1}$ (aCH_2) can be attributed to the concentration of lipids in the lipid matrix. The bandwidth of a vibration is connected to the structural changes of the lipids. Usually, an increase in bandwidth means an increase in the interaction between functional groups.

3. Effect of Treatment Time

The decreases in absorbance in the symmetric and asymmetric methylene stretching bands and in the symmetric methyl stretching band indicate the extraction of lipids, with saturation after 120 s of the treatment (Fig. 5).

Tsai et al. found that the absorbance of the CH_2 asymmetric stretching band correlates with the lipid amount better than the CH_2 symmetric stretching band.⁴¹ However, the ratio of the asymmetric stretches of CH_3 and CH_2 correlate with the lipid amount in the stratum corneum even better (Fig. 6, $\text{aCH}_2/\text{aCH}_3$ ratio).⁴²

Lipids in the stratum corneum are composed of long hydrocarbon chains. The lipid tails consist of CH_2 groups with CH_3 groups at the end of the chains. As the number of

FIG. 5: Symmetric and asymmetric stretches/absorbance of methyl and methylene

FIG. 6: Ratio of symmetric and asymmetric stretches of methyl and methylene. Ratio aCH_2/aCH_3 represents evolution of number of lipids in stratum corneum; sCH_2/sCH_3 represents evolution in length of lipid chains or decrease in number of CH_2 groups by oxidation.

CH_3 functional groups is proportional to the absorbance of the symmetric stretches of CH_3 , and the number of CH_2 functional groups is proportional to the absorbance of the symmetric stretches of CH_2 , the ratio of these bands can characterize the average length of the lipid chains (Fig. 6, sCH_2/sCH_3 ratio).

When this ratio changes, the length of the hydrocarbon chain also changes. In our situation, the change in the ratio indicated the breaking of bonds because of the plasma interaction (ions, radicals) with the lipids, or the decrease in CH_2 functional groups because of oxidation.

According to Fig. 6, decrease in the number of CH_2 functional group and lipid extraction stopped after 120 s of treatment. The same saturation effect was observed in the bandwidth in the symmetric methylene stretching band (Fig. 7). The bandwidth increased from 16 cm^{-1} to almost 20 cm^{-1} . This means that the intermolecular interaction between lipid chains increased, suggesting a disturbed lamellar structure.

Plasma's influence results from any of several phenomena, such as temperature, UV radiation, electric field, and presence of ions and radicals. To find out which phenomenon was responsible for changes in the pig skin samples, we investigated separately the effect of temperature and UV radiation.

4. Temperature Effect

The rise in temperature of the samples caused by plasma irradiation and hot air flow from the fan during 60-s treatment was measured and compared by a thermal infrared

FIG. 7: Dependency of bandwidth of methylene symmetric stretch on treatment time (black square) and on temperature (solid circles)

camera (TVS-200, Avio). The change in temperature is shown in Fig. 8. The hot air flow trials reached maximal temperatures of 40°C, 48°C, and 75°C with decreasing distance while the skin treated by plasma jet coupled with 4 kV reached a maximum temperature of 45°C (red open circles in Fig. 8). After plasma treatment, the temperature decreased to the initial value after approximately 100 s. The skin samples were examined via ATR-FTIR after the decrease in temperature. Heating can increase the skin's fluidity, which has been demonstrated several times.^{42,43}

The structural changes of the stratum corneum lipids occurred between 20°C and 40°C, changing from an orthorhombic to a hexagonal order.⁴⁴ However, as the temperature decreased, the lipid membrane returned to its original state. Figure 7 (solid circles) demonstrates the evolution of the bandwidth of the CH₂ symmetric stretching band after treatment with heated air flow at different maximal temperatures. The heat treatment had no influence on bandwidth after the skin cooled. A slight increase was observed at a maximal temperature of 75°C, but this temperature is not suitable for skin treatment. These results showed that plasma temperature below 45°C does not affect the skin significantly.

5. UV Radiation Effect

Plasma is a source of radiation generated by excited species. Argon plasma ignited in the air atmosphere emits light in the range of 280–450 nm by the nitrogen (C-B) second

FIG. 8: Evolution of skin during plasma jet treatment (open red circles) and during heating by hot air (solid circles)

positive system, in the range of 306–322 nm by the OH(A-X) system, in the range of 190–290 nm by the NO-gamma (A-X) system, in the range 130–200 nm by the nitrogen (A-X) LBH system, and at 109 and 126 nm the first and second continuum of the argon dimer. To investigate the effect of UV radiation, we irradiated the skin samples with plasma jet through a filter. However, emission under 170 nm was very strongly absorbed by molecular oxygen and partially by nitrogen. Transmittance through the filter materials is shown in Fig. 9. We also used a lamp with an emission of 254 nm and another lamp with an emission of 365 nm. The irradiation of a sample with visible light should have almost no effect. However, neither UV (through the BK7 or the MgF₂ filter) had any noticeable effect on the structure of the skin lipids after 1 min. In our case, no effect of UV/VUV radiation on the bandwidth of the lipid bands was observed, probably because of the short irradiation duration.

UV radiation can cause the formation of epoxides, hydroxides, hydrogenation of the double bonds, and breaking down of carbon chains.⁴⁵ Mainly fatty acids with double bonds are sensitive to the formation of oxygenated molecules in the presence of oxygen in the air.

The arrangements of stratum corneum lipids and water loss prevention recovered after three days and returned to initial state, thanks to the skin's repair processes.⁴⁶ Trommer et al.⁴⁷ found that the peroxidation of unsaturated fatty acids and cholesterol is limited and not linearly proportional to time because the difference between irradiation by UV at 30 min and 24 h was only twofold.

FIG. 9: Transmittance of PEN film, borosilicate glass (BK7), and MgF_2 glass used for investigation of UV radiation effects on skin lipids

6. Posttreatment Behavior

We observed changes in the symmetric methylene stretching band in the stratum corneum in skin samples after 10 min, 1 day, 2 days, and 5 days after plasma jet treatment (Fig. 10 black and red or everyday treated lines), and in skin samples not treated by plasma jet (Fig. 10 blue or non-treated line). The skin samples treated for 1 min (Fig. 10 black line), and the skin samples treated every day for 1 min (Fig. 10 red or everyday treated line) were observed. Some delay-induced changes were seen. Points at time zero represent the samples before plasma treatment (Fig. 10 red or everyday treated lines and black lines). Decreasing bandwidth is usually determined by strengthening of the barrier

FIG. 10: Bandwidth of symmetric stretching band of CH_2 of nontreated skin samples, one-time treated sample and once daily 1-min-treated sample

properties and vice versa. The nontreated samples showed decreasing bandwidth (Fig. 10 blue or non-treated line). Because the nontreated skin was stored at the room temperature, this decrease was related to structural changes due skin aging and water loss. If the skin was treated by plasma jet once, the bandwidth increased (from approximately 16 to 18 cm^{-1}). After one day, we observed that the lipid membrane returned to the initial state, and by the second day the bandwidth reached initial values. However, when the sample was treated every day for 1 min, the bandwidth was maintained higher for a longer time.

IV. CONCLUSION

Argon plasma jet treatment of the stratum corneum was investigated using ATR-FTIR. The treatment was applied at 10 s up to 900 s. Our work focused on the bandwidth and the absorbance of the symmetric and asymmetric stretching CH_2 and CH_3 bands and their ratios. All of the main changes in the skin occurred up to 120 s; after this time, saturation was observed. We conclude that the decrease in absorbance of the lipid vibrations in the wavenumber range of 2,800–3,000 cm^{-1} was induced by lipid extraction. The etching of the stratum corneum by plasma jet decreased in thickness from 30 to 10 μm in 60 s. The amount of intercellular lipids per layer decreased with increasing depth from the surface of the stratum corneum. Thus, the fractional volume occupied by the corneocytes increased with increasing depth.^{48–50} We observed that UV radiation and temperatures up to 45°C did not cause any long-term change in lipid structure, as characterized by the symmetric stretching band of CH_2 . We conclude that etching, lipid extraction, and structural changes in the lipids were caused by particles present in the plasma such as ions, electrons, metastable particles, or radicals. However, we could not estimate the effect of the electrical field. The increase in bandwidth lasted up to two days. This time could be increased by repeated plasma treatments. Our study of the structural changes in lipids caused by plasma treatment will contribute to improved transdermal drug delivery and identification of the risks of skin sterilization.

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