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Original Article

# Electron spectroscopic analysis of the human lipid skin barrier: cold atmospheric plasma-induced changes in lipid composition

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**Abstract:** The lipids of the stratum corneum comprise the most important components of the skin barrier. In patients with ichthyoses or atopic dermatitis, the composition of the skin barrier lipids is disturbed resulting in dry, scaly, itching erythematous skin. Using the latest X-Ray Photoelectron Spectroscopy (XPS) technology, we investigated the physiological skin lipid composition of human skin and the effects of cold atmospheric plasma treatment on the lipid composition. Skin lipids were stripped off forearms of six healthy volunteers using the cyanoacrylate glue technique, plasma treated or not and then subjected to detailed XPS analysis. We found that the human lipid skin barrier consisted of 84.4% carbon (+1.3 SEM%), 10.8% oxygen (+1.0 SEM%) and 4.8% nitrogen (+0.3 SEM%). The composition of physiological skin lipids was not different in males and females. Plasma treatment resulted in significant changes in skin barrier lipid stoichiometry. The total carbon amount was reduced to 76.7%, and the oxygen amount increased to 16.5%. There was also a slight increase in nitrogen to 6.8%. These changes could be attributed to reduced C-C bonds and increased C-O, C=O, C-N and N-C-O bonds. The moderate increase in nitrogen was caused by an increase in C-N and N-C-O bonds. Our results show for the first time that plasma treatment leads to considerable changes in the human skin lipid barrier. Our proof of principle investigations established the technical means to analyse, if plasma-induced skin lipid barrier changes may be beneficial in the treatment of ichthyotic or eczematous skin.

**Key words:** cold atmospheric plasma – epidermal barrier – skin lipids – X-Ray Photoelectron Spectroscopy

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## Introduction

The lipids of the stratum corneum comprise the main components of the hydrophobic human skin barrier regulating water homoeostasis (1,2). The epidermal lipid barrier film is composed of a more or less equimolar mixture of ceramides, cholesterol and long-chain free fatty acids. Seven different ceramides belonging to the class of sphingolipids can be discerned (2) and especially the ceramide profile may be important in relation to the barrier function of the skin. In general, there is quite limited data on the physiological lipid profile of human skin (3), for example, in relation to age or gender. Increased age may lead to an overall reduced lipid content in human skin (4,5). Jungersted et al. (1) found that a higher ceramide/cholesterol ratio was present in men compared with women. Reduced amounts of ceramide 3 were found to be associated with increased transepidermal water loss, a marker for a disturbed skin barrier function (6,7). More recently, the notion that mutations in the filaggrin gene result in autosomal-dominant ichthyosis vulgaris (8) and are associated with atopic eczema (9) – both diseases with severe defects in skin barrier function – has fostered interest in research of normal human skin lipid barrier and changes of skin lipids in diseased skin. For example, patients with atopic eczema and common filaggrin mutations exhibited different amounts of the ceramides 1, 4 and 7 compared with healthy controls. Interestingly, these patients also exhibited higher skin pH values and a higher erythema rate (10).

In cold plasma medicine, a new field, anti-inflammatory, anti-itch, anti-microbic, UV and other therapeutic modalities are combined within one plasma (ionized air) treatment (11–15).

Published cold plasma applications include successful treatment of atopic eczema as well as other pruritic diseases (12,13,16). Generally, two types of cold plasma can be discerned: direct plasma (dielectric barrier discharge – DBD, corona discharge) and indirect plasma (plasma torch, plasma jet). While dielectric barrier discharge (DBD), plasma treatment of technical surfaces is a standard method since years, the DBD plasma treatment of biological tissue is quite novel (17,18). DBD generates a low-temperature plasma under atmospheric pressure and, thus, is a suitable instrument for a non-destructive treatment of biological material (19). For our investigation, a non-equilibrium, weakly ionized physical DBD plasma is generated by the application of high voltages across small gaps, whereas the electrode is covered by a dielectric. This

non-conducting layer avoids the transition of the gas discharge into a hot arc by limiting the current. The biological tissue itself (i.e. skin lipids) acts as the second electrode. The plasma generator induces pulses with amplitudes reaching up to 13.5 kV (16,20). Of note, we also established already that plasma treatment reduces pH values on human lipid films indicating the potentially normalizing effect of plasma treatment on skin lipids in eczema or ichthyosis patients (18).

In the last decades XPS, respectively electron spectroscopy for chemical analysis (ESCA), has developed into a powerful tool for the investigation of surfaces of biomaterials (21–23). With this technique, it is possible to detect the elemental composition of material surfaces and furthermore to assign functional groups present on these surfaces. Therefore, XPS provides a detailed insight into surface chemistry. For example, XPS was used to investigate the serum protein adsorption on eukaryotic cells and to analyse the fouling of HEMA-based contact lenses (24,25). With respect to dermatological research, M.K. Bahl studied the removal of skin lipids by solvents and surfactants with ESCA in 1985 (26). In 1987, E. D. Goddard and W. C. Harris investigated the adsorption of polymers and lipids on stratum corneum membranes (27). Finally, Y. Chen and T. S. Wiedmann have used XPS in 1996 in combination with atomic force microscopy (AFM) to get insight into the molecular structure of the lipids in the corneal layer of the epidermis itself (28). Since then, XPS techniques significantly improved until today allowing a more detailed and reliable analysis of chemical structures (21,23).

In this preliminary study, we applied the latest XPS techniques to assess the effects of cold plasma treatment on human skin barrier lipids. Lipids from six healthy volunteers were stripped off forearms onto sample holders and then analysed via X-Ray Photoelectron Spectroscopy with and without prior plasma treatment. A distinct and reproducible change in stratum corneum lipid chemistry could be detected after plasma treatment.

## Methods

### Probands

Four male and two female probands (age range 25–50 years) were recruited for this pilot study, all employees of the Institute of Energy Research and Physical Technologies, Clausthal University of Technology and the Faculty of Natural Sciences and Technology, University of Applied Sciences and Arts Hildesheim/Holzminden/Göttingen. The study was performed according to the principles of the Declaration of Helsinki and the institutional review board of the University Medical Center in Göttingen. All probands gave their informed consent orally as well as in writing.

### Lipid sample preparation

The skin lipid samples were obtained by stripping off skin lipids from the inside of the forearm as described in the literature (28,29). The forearm was shaved and washed with isopropanol previously. Then, a stainless steel sample holder with a diameter of 14 mm and a droplet of cyanoacrylate skin glue (MedLogic LiquiBand, Plymouth, UK) was pressed for 2 min onto the arm until the glue almost dried. Then, the sample holder was stripped off. Part of the skin barrier lipids were subjected to plasma treatment before transferring them into a vacuum chamber for XPS analysis.

### Cold atmospheric plasma

The application of plasma on biological samples was possible through the realization of DBD at atmospheric pressure. The dielectric barrier in the gas gap causes a self-inflicted extinction of the discharge resulting in short discharge times. Thus, the displacement current is limited and the gas temperature keeps approximately ambient temperature. These were necessary assumptions for a non-destructive treatment of biological tissue. The biological effective plasma components during a plasma exposure are UV emission, displacement currents, temperature and reactive species. This mixture could be used for sterilization of medical equipment by generating a lethal environment for germs, for example, for staphylococcus epidermidis (30). Another effect of plasma on biological samples was the acidification of lipid film surfaces for up to 2 h after plasma treatment. A couple of skin diseases go hand in hand with changes in pH value, hence this fact might be utilized in dermatology (18).

### Plasma treatment of lipids

The plasma treatment in this work was performed under atmospheric conditions. The distance between the lipid sample and the electrode was adjusted to 1 mm. The sample was treated for 45 s. The DBD was driven using a pulsed power supply at a peak-to-peak voltage of 15 kV with a voltage pulse duration of 70 $\mu$s and a pulse repetition rate f of 300 Hz. The plasma power was determined to be 150 mW (±10 mW) by applying the Lissajous figure method (18). The electrode had a diameter of 8.5 mm, so the average power density can be calculated to 269.6 W/cm² (±18 W/cm²). A treatment time of 45 s means that the lipid was exposed with an energy density of 12.1 kJ/cm² (±0.8 kJ/cm²). The gas discharge was ignited in ambient air. The gas temperature was identified to amount to 300 K, that is, approximately. ambient temperature.

### XPS analysis

An ultrahigh vacuum chamber with a base pressure below $5 \times 10^{-10}$ mbar which has been described in detail previously (31) was used to carry out the spectroscopic measurements. Electron spectroscopy was performed by a hemispheric analyser in combination with a commercial non-monochromatic X-Ray source (Fisons XR3E2-324; VG Microtech, Uckfield, UK). During XPS, X-ray photons hit the sample surface under an angle of 85° to the surface normal illuminating a spot of several mm in diameter. For all measurements presented here, the Al Kα line with a photon energy of 1486.6 eV was used. Electrons are recorded by the hemispherical analyser with an energy resolution of 1.1 eV under an angle of 5° to the surface normal. All XP spectra are displayed as a function of binding energy with respect to the Fermi level.

For quantitative XPS analysis, photoelectron peak areas were calculated after Shirley background correction. With this correction, we achieved the most consistent stoichiometric results for our measurements. Peak fitting with Gauss-type profiles was performed using OriginPro 7G including the PFM fitting module which uses Levenberg–Marquardt algorithms to achieve the best agreement possible between experimental data and fit. Photoelectric cross-sections as calculated by Scofield (32) and inelastic mean free paths from the NIST database (33) as well as the transmission function of our hemispherical analyser were taken into account when calculating stoichiometry. Essentially, the peak fitting procedure was performed as described in (34). At least three analyses

were performed per proband with and without plasma treatment, respectively.

## Results

### Experimental preconditions

As mentioned previously, stratum corneum lipids were stripped off forearms from the six healthy volunteers onto sample holders using the cyanoacrylate glue technique. The lipid probes on the sample holders were plasma treated or not and then subjected to XPS analysis.

To ensure that the used glue does not interfere with our analyses, we performed preliminary XPS experiments on pure glue probes. Due to chemical elements which are part of the glue but not human lipids or the sample holder, we could clearly distinguish the spectral contributions from the glue and the sample holder. The results also revealed that the skin lipid probes obtained were thick enough to exclude any influence of the glue (data not shown).

The interpretation of XPS data for skin lipids is quite challenging due to the large number of components. In addition to cholesterol, the basic structure of the stratum corneum lipids consists of ceramides and free fatty acids. We correlated these three components with the binding states of the lipid probes under the prediction that we only detect these three components and compared our measurements to previous works (27,35–37). Based on these procedure, we were able to fix binding energies, distances between the Gaussian's curves and also full widths of half maximum (FWHM) for all following measurements which finally led to considerably reproducible results.

### Physiological skin lipid composition

Figure 1 shows the typical XPS survey spectra of the untreated (black solid line) and plasma treated (red solid line) physiological human lipid film. For the untreated film, we identified the photoelectron peaks at a binding energy of around 530 eV and Auger structures of oxygen in the range from 950 eV to 1050 eV as well as a carbon peak between 275 eV and 295 eV. Furthermore, we identified a small amount of nitrogen at the binding energy around 399 eV. We did not detect any parts of the stainless steel sample holder nor of the glue in the spectra. The investigated untreated skin lipid probes contained a mean amount of 84.4% carbon (+1.3 SEM%), 10.8% oxygen (+1.0 SEM%) and 4.8% nitrogen (+0.3 SEM%). The composition of physiological skin lipids was not different in males [84.4% carbon (+1.3 SEM%), 10.8% oxygen (+1.0 SEM%) and 4.8% nitrogen (+0.3 SEM%)] and females [85.1% carbon (+1.3 SEM%), 9.7% oxygen (+1.2 SEM%) and 5.1% nitrogen (+0.3 SEM%)]. An influence of the gender and the donor age on the physiological skin lipid composition could be not observed in the present study.

The upper spectrum in Fig. 2a shows the detailed XPS spectra of the carbon C 1s peak of untreated skin lipids. The obtained data are plotted as black dots the mathematical fit is displayed as a solid red line. The single Gaussian curves are displayed in solid blue lines. A Shirley background correction was used. Based on our experimental preconditions, the distance between the structures at 285.0 eV, 286.2 eV and 287.4 eV in each case was set to 1.2 eV. Furthermore, the distance between the structures at 287.4 eV and 288.7 eV was set to 1.3 eV. The total value of the Gaussian curve at 285.0 eV and the structure at 282.8 eV were set to a global value of 70%. Considering the stoichiometry, this

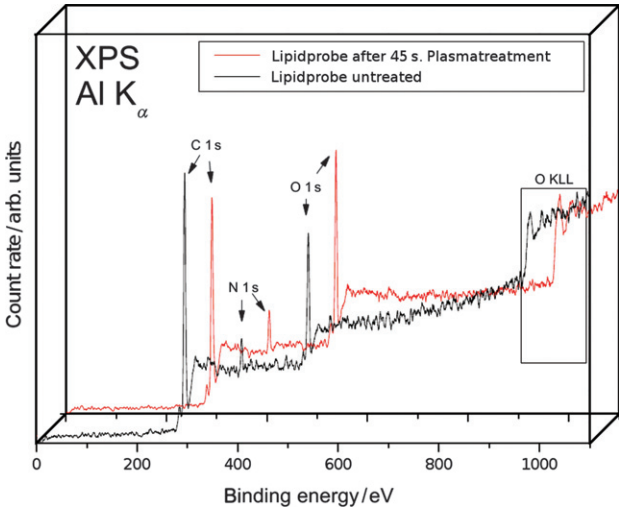


**Figure 1.** X-Ray Photoelectron Spectroscopy survey spectrum of untreated (black solid line) and plasma-treated (red solid line) skin probe.

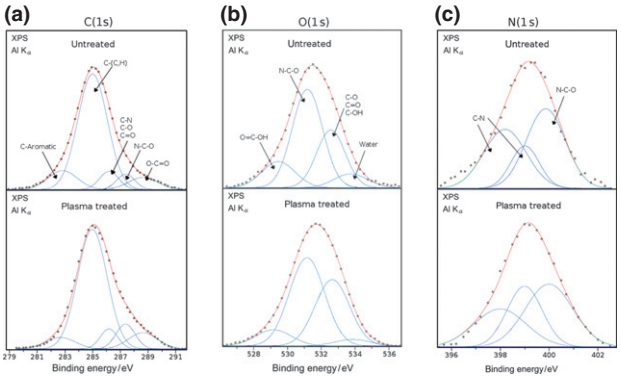


**Figure 2.** (a) X-Ray Photoelectron Spectroscopy C 1s region of the untreated and plasma-treated skin probe. (b) XPS O 1s region of the untreated and plasma-treated skin probe. (c) XPS N 1s region of the untreated and plasma-treated skin probe.

relates to the expected portion of carbon not bound to nitrogen or oxygen. The first peak at 282.8 eV had a FWHM of 2.5 eV and most likely relates to aromatic carbonates. The second structure with a binding energy of 285.0 eV and a FWHM of 2.5 eV could clearly be assigned to C-C bonds. The third Gaussian curve at 286.2 eV with a FWHM of 1.5 eV corresponds to C=O, C-O and C-N bonds. The peak around 287.4 eV with 1.6 eV FWHM is related to N-C-O bonds. O-C=O bonds are displayed at the binding energy of 288.7 eV with a FWHM of 2.5 eV.

Figure 2b depicts the detailed XPS spectra (upper one) of the oxygen O 1s peak of untreated skin lipids. Again, a Shirley background correction was used. The first Gaussian curve corresponds to O=C-OH bonds and was preset in the region between 529 eV up to 530 eV with a FWHM of 2.5 eV. The structure at 531.5 eV and a FWHM of 2.4 eV which belongs to N-C-O bonds was set to the same fraction at the global stoichiometry as the N-C-O peak in the carbon detail spectra. The distance to the third peak at the binding energy of 533.0 eV and the FWHM of

2.3 eV relating to the C-O, C=O and C-OH bonds was retrained at 1.5 eV. The fourth structure at a binding energy of 533.9 eV and a FWHM of 2.5 eV in Fig. 2b belongs to retained water (38).

Figure 2c shows the detailed XPS spectra (upper one) of the nitrogen N 1s peak of untreated skin lipids. As the binding energies of many nitrogen bonds are indistinguishable, the interpretation of the spectra is quite challenging. The most reliable results for all data were obtained predicting that the first two peaks at 398.0 eV and a FWHM of 2.5 eV and at 399.0 eV and a FWHM of 1.7 eV correspond to C-N bonds. The third structure at 400.0 eV with a FWHM of 2.3 relates to N-C-O bonds.

**Changes in skin lipid composition after plasma treatment**

When the two XPS spectra with and without plasma treatment of skin lipids displayed in Fig. 1 are compared a clear change in skin barrier lipid stoichiometry is visible. This indicates that plasma treatment had a clear effect on skin barrier lipid composition. On the one hand, we found an overall reduction of carbon and on the other hand an overall increase of oxygen and a small increase of nitrogen. The investigated plasma-treated skin lipid probes contained a mean amount of 76.7% carbon (+0.7 SEM%), 16,5% oxygen (+0.4 SEM%) and 6.8% nitrogen (+0.3 SEM%).

For the plasma-treated samples, we applied the same basic analysis of the detail XPS spectra as for the untreated lipid probes. Again, the mathematical fit was in an excellent agreement with the measured data. As shown in Fig. 2a (bottom spectra), aromatic carbonate as well as C-C bonds were reduced, whereas C-O, C=O, C-N and N-C-O bonds partly increased. There was no change in the rate of the O-C=OH structure. Figure 2b shows that we observed only a small reduction in the retained water ratio after plasma treatment (bottom spectra). N-C-O bonds and C-(O; OH), C=O bonds clearly increased. O-C=OH bonds remained unchanged. The moderate increase in nitrogen depicted in Fig. 2c was caused by an increase in C-N and N-C-O bonds.

## Discussion

Generally, the stoichiometry of the untreated physiological skin barrier lipid composition that we observed was in good agreement with previously published results from Goddard and Harris (27) and Chen and Wiedmann (28). In line with our results, the few available XPS investigations of skin lipid composition showed a higher oxygen and nitrogen content as one would expect for skin surfaces which solely contain ceramides, free fatty acids and cholesterol. Goddard et al. showed that washing the skin with sodium dodecyl sulphate increased the oxygen and nitrogen content even more (27). The authors attributed this to an increase of proteins resulting in a more keratinized surface. Chen et al. also explained the higher nitrogen content with the presence of proteins in addition to lipids at the skin surface (28). In our study, the skin was washed with isopropanol prior to the analysis with XPS. Thus, we also assume that our stripped off skin samples consisted not only of lipids but also proteins, too. In addition, using our procedure of sampling, we found at first glance nearly the same stoichiometry independent from the skin donor (i.e. here independent of gender). This was revealed using skin samples from different volunteers as well as multiple measurements per donor. Therefore, our clear and reproducible results on the physiological composition of the human skin lipid barrier are an excellent basis to further investigate the individual components of the stratum corneum in more detail as well as the influence of cold plasma treatment on the molecular composition of the skin barrier using XPS.

After cold plasma treatment under atmospheric conditions, the XPS survey spectrum showed a strong increase in oxygen as well as a decrease in carbon in the samples investigated. The main change in the structure of the skin lipid probe after plasma treatment was the increase of C-O and C=O bonds, whereas at the same time, the C-C bonds in the carbon spectra were reduced. The global stoichiometrical portion of the C-O and C=O in the oxygen O 1s spectra rose from 3% to 6%. A possible explanation for this increase of the oxygen global stoichiometry is that carbon bonds broke related to the integration of oxygen radicals during the plasma treatment. A comparable effect of oxygen enrichment was shown in the work of L. Wegewitz et al. after plasma-induced oxidation of germanium (39) and by L. Klarhöfer et al. after plasma treatment of lignin and cellulose (31).

After plasma treatment, we also found a small increase of the nitrogen N 1s structure in combination with an increase of the N-C-O portion in the carbon and in the nitrogen detail peaks. This may be explained by enrichment of nitrogen at the outer side of the lipid sample. An integration of nitrogen from outside due to plasma discharge can be excluded as referenced by L. Wegewitz et al. (39).

In summary, we identified for the first time a fundamental and reproducible influence of cold atmospheric plasma treatment on the human skin lipid barrier system as can be especially derived from the clear structural changes in the oxygen detailed spectra. Our experimental setting and results pave the way for more detailed fundamental analyses of the effects of plasma treatment on the human skin lipid barrier deciphering effects on each of the three main lipid components. Especially, plasma-induced distinct changes in the ceramide composition would be interesting to investigate as this composition is disturbed in ichthyosis and atopic eczema patients (40) and may be reverted by plasma treatment. The glue-stripping technique is a standard technique to obtain skin lipids for further analysis as referenced. As the plasma treatment is a topical treatment, the XPS technique has a vertical information depth of only few millimetres, and we established that neither the glue nor the sample holder interferes with the XPS analysis, we are confident that our results obtained *in vitro* are comparable with the changes on the outer most skin lipids that would occur after direct plasma treatment of the skin. Cold plasma treatment can be applied as a topical skin treatment without skin contact between the plasma device and the skin. The air between the device and the skin (approximately 1–2 mm) is ionized, and this plasma acts onto the skin. We just completed a clinical trial in which we treated venous leg ulcers with cold plasma. This is the first clinical trial officially listed in trial databases like 'clinical trials.gov' and approved by BfArM and ethics according to actual legislation (41).

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## Author contribution

Marcel Marschewski, Joanna Hirschberg, Tarek Omairi performed the experiments and obtained the data. Oliver Höfft, Wolfgang Viöl, Steffen Emmert, and Wolfgang Maus-Friedrichs designed the study, analysed or interpreted the data, wrote or critically revised the manuscript.

## Conflict of interests

The authors have declared no conflicting interest.

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