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Effects of the Effluent of a Microscale Atmospheric Pressure Plasma-jet Operated with He/O_2 Gas on Bovine Serum Albumin

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ABSTRACT: Cold atmospheric pressure plasmas (CAPs) are being investigated for medical applications, and the first clinical studies are promising. However, interactions between plasmas and biological samples are only partly understood on a molecular level. In this study, bovine serum albumin (BSA), a standard model for plasma-mediated etching of biological samples, was used to investigate the effects of different components of an He/O_2 plasma effluent on proteins. The X-jet features an optional lateral helium flow that splits the plasma effluent into particles and (V)UV radiation. BSA samples were exposed separately to plasma-emitted particles, UV radiation, or the combination of both. Afterward, plasma-treated samples were investigated using SDS-PAGE and western blot analysis for amino acid strand breaks, but none were detected. Furthermore, treated samples were investigated by Raman spectroscopy to search for chemical modifications. We found that treatment with the X-jet has little effect on BSA. Minor changes in the Raman spectra suggest modifications of tyrosine residues and some degree of oxidation of sulfur-containing amino acids. Our findings suggest that for the X-jet effluent, etching is the main effect of plasma on BSA, making BSA a suitable model with which to study protein etching.

KEY WORDS: Atmospheric pressure plasma, X-jet, protein modification, BSA, Raman spectroscopy

ABBREVIATIONS

a-C:H: amorphous hydrogenated carbon layer, a.u.: arbitrary unit, BSA: bovine serum albumin, CAP: cold atmospheric plasma, DBD: dielectric barrier discharge, GapDH: glyceraldehyde 3-phosphate dehydrogenase, PVDF: polyvinylidene fluoride, ROS: reactive oxygen species, SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

I. INTRODUCTION

Cold atmospheric plasmas (CAPs) are employed in the emerging field of plasma medicine to deactivate microorganisms and to treat wounds.[1] It is known that CAPs are able to deactivate microorganisms on very short time scales both under laboratory conditions and in clinical settings.[2,3] Not only living organisms are deactivated by

CAPs, but also different biological macromolecules.[4] Bovine serum albumin (BSA) is the model protein commonly used to investigate plasma-based etching of biological samples.[4–6]

BSA and the homologous serum albumins of other organisms are the most abundant proteins in blood. They deliver fatty acids and other water-insoluble molecules to their destinations.[7] Mature BSA has a calculated molecular weight of ~66 kDa and features a total of 35 cysteines, only one of which has a free thiol group. The others form 17 intramolecular disulfide bonds contributing to BSA stability.[8]

BSA has been used to study etching with both atmospheric and low-pressure sources. Etching is the physical removal of matter. It is caused by ions and metastables with high potential energy levels present in plasma.[9] Different methods for determining etching rates have been described in the literature.[5,10] For most plasmas, etching rates can be modulated depending on whether or not etching is desired. The question remains: Which effects other than etching occur when proteins are subjected to plasma treatment? It is known that plasma can introduce different chemical modifications into biomolecules, depending on the employed plasma and the sample.[4,6,11,12] In proteins, the introduction of chain breaks has been demonstrated by subjecting soluble BSA to DBD treatment.[13] Treatment of proteins with the X-jet, a modified microscale atmospheric pressure plasma-jet, has been shown to cause a loss of function in proteins with catalytically active cysteines such as glyceraldehyde 3-phosphate dehydrogenase (GapDH).[4] The relevance of plasma-mediated protein modifications in plasma-exposed patient tissue and the microflora is unknown.

In this study, we investigated the influence of different effluent components emitted by an He/O_2 plasma, namely reactive oxygen particles and (V)UV photons, on BSA. The X-jet and its effluent are well-characterized and have been described elsewhere.[14,15] In this study, we used the X-jet version described in Lackmann et al., which consists of three independent-jets, each with a specific nozzle geometry to allow sample treatment with the different effluent components.[4] The UV-jet features an additional He flow to steer the heavy particles into a second channel by convection, whereas the emitted photons are unaffected by the steering flow and propagate in a straight line. The nozzle of the particle-jet is bent twice such that photons are prevented from reaching the sample. The complete-jet features a straight nozzle permitting both photons and particles to reach the sample. In all three nozzles, the distance between the plasma and the nozzle exit is 8 mm, and the distance from the plasma to the sample is always 12 mm. The three-jets were operated with He gas and a 0.6% O_2 admixture inside an He-flooded chamber with 100 mbar overpressure as described elsewhere.[4] Treating the samples under an He atmosphere minimizes the interference of air with regard to absorption of emitted photons and generation of air-derived reactive species (Fig. 1).

We previously showed that BSA etching rates of the emitted particles resemble those for an amorphous hydrogenated carbon layer (a-C:H layer). In contrast, no etching of either BSA or a-C:H layers was observed after treatment with the UV-jet.[6] In the present study, we investigated the integrity of the protein backbone and chemical modifications

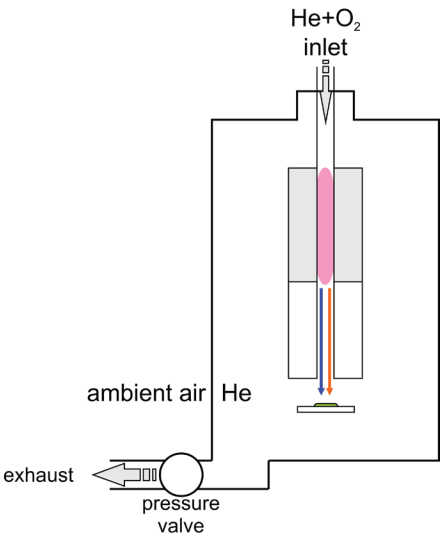

FIG. 1: Schematic representation of the environmental He chamber The X-jet array is located inside an environmental chamber (only one-jet shown for demonstration purposes). The pressure inside the chamber is 100 mbar higher than the ambient pressure to prevent influx of ambient air through the exhaust, which is necessary to keep the pressure inside the chamber stable with the help of an adjustable pressure valve. Samples (green) are placed inside the chamber in a fixed distance (4 mm) to the different jets.

of the protein using denaturing polyacrylamide gelelectrophoresis (SDS-PAGE), western blot analysis, and Raman spectroscopy.

II. METHODS

A. Sample Preparation and Treatment Conditions

A 10 mg/ml stock solution of BSA (AppliChem) was prepared and stored in aliquots at –20°C. Before treatment, the stock solution was diluted to 1 mg/ml, and 10 µl aliquots were spotted on glass slides in two 5 µl portions to yield a maximal spot diameter of 3 mm. Samples were dried under a clean bench for 2 h before treatment. Treatment with the X-jet was performed in an environmental He chamber. Prior to sample treatment, the chamber atmosphere was exchanged for He, generating 100 mbar overpressure to prevent influx of ambient air through the outlet valve. The pressure was kept constant during treatment. The X-jet was driven at 13.56 MHz with 230 V_{RMS} with 1.4 slm He (5.0 purity) as the feed gas and 8.4 sccm O_2 (4.8 purity) admixture. In the X-jet, the electrodes are 1 mm apart and the plasma is ignited in a 30-mm-long channel. Three identical-jets with different nozzles are mounted next to each other and allow treatment of samples with the plasma effluent (complete-jet), only the emitted (V)UV photons (UV-jet), or only the plasma-emitted particles (particle-jet).[4]

B. SDS-PAGE

Plasma-treated samples were washed off the glass slides with 20 µl 40 mM KH_2PO_4 buffer (pH 7.4), and protein concentrations were determined by Bradford assay using the Roti-Quant solution (Roth). Of each sample, 100 ng protein were boiled in protein running buffer for 10 min and loaded onto discontinuous 10% polyacrylamide gels. Gels were run at 80 V for 30 min followed by an additional 90 min at 120 V. Afterward, gels were stained with Coomassie R-250.[16] The Benchmark Protein Ladder (Invitrogen) was used as a protein size marker.

C. Western Blotting

Samples were prepared, treated, and separated by SDS-PAGE as described above. Instead of Coomassie staining, proteins were blotted with a semi-dry blotting system (Trans-Blot SD, Bio-Rad) onto PVDF membranes (Roti-PVDF 0.45 µm pores, Roth) following the instruction manual. Membranes were blocked with skim milk prior to detection of BSA with a monoclonal anti-BSA antibody produced in rabbit (Sigma-Aldrich) and an anti-rabbit secondary antibody produced in goat (Sigma-Aldrich) by measuring luciferase fluorescence (Luminata Forte, Millipore) with the FluorChem SP camera system (Alpha Innotech). A prestained protein size marker (Page Ruler plus prestained, Peqlab) was used as a molecular weight marker.

D. Densitometry of SDS-PAGE and Western Blot Signals

To quantify signal intensity, high-resolution scans of SDS-PAGE gels and blotted membranes were analyzed with Alpha EaseFC densitometrically (Version 4.0.0, Alpha Innotech). Background correction was performed for each individual signal by choosing an appropriate reference area and subtracting the background signal intensities.

E. Raman Spectroscopy

Confocal Raman spectroscopy was performed using a WITec alpha300 RAS microscope (WITec). A single-frequency diode laser with a wavelength of 785 nm was used for excitation with a power of 100 mW. The laser beam was coupled into the microscope through a single mode optical fiber and then focused on the sample surface with a 100× objective (NA = 0.9). The Raman scattered light was collected with the same objective and registered by a back-illuminated charge-coupled device (CCD) detector (128*1024 pixels, kept at –60°C) after passing a multimode fiber (100 µm diameter) and a diffraction grating (300 grooves per mm). Raman spectra were taken with a total integration time of 60 s for each spectrum (2.5 s, 24 acquisitions) and a wave number resolution of +/–2 cm^{-1}. Samples were measured at room temperature. Raman data sets were managed using WITec Project 2.10 (2012). For background subtraction, a polynomial fit was calculated using points defined in an area of the spectrum with no visible bands prior

to user-defined cosmic ray removal. Hydrogen peroxide–treated samples were used as an oxidation control to verify the possible oxidation of cysteines and disulfide bonds in BSA. Aliquots of 20 µl BSA solution (1 mg/ml) were incubated with 5% hydrogen peroxide for 20 min at 40°C before spotting 10 µl in two consecutive steps onto glass slides, dried, and investigated using Raman spectroscopy.

III. RESULTS AND DISCUSSION

BSA has previously been shown to be subject to etching when treated with the X-jet.[6] To investigate whether etching involves formation of intermediates with fragmented amino acid chains, the size of plasma-treated BSA was investigated using SDS-PAGE.

A. SDS-PAGE and Western Blot Analysis

Samples were treated with the UV-jet, the particle-jet, or the complete-jet for 60 s or 600 s, and equal amounts of BSA were then subjected to analysis by SDS-PAGE (Fig. 2A). BSA has a calculated molecular weight of approximately 66 kDa. The occurrence of significant amounts of strand breaks results in the loss of signal intensity at 66 kDa. In addition, new signals are indicated if the protein backbone breaks at specific hotspots.

Densitometrical analysis revealed no significant loss in band intensity compared to the control (treated with gas only). The controls showed a background-corrected intensity of 51 ± 4 arbitrary units (a.u.) per pixel, whereas UV-jet–treated samples had an averaged pixel intensity of 52 ± 2 a.u. Samples subjected to the particle-jet or the complete-jet both had a slightly reduced intensity of 48 ± 1 a.u. per pixel. However, based on the experimental variation (see deviation between control samples), the loss of intensity does not seem to be significant. In addition, no appearance of new bands compared to the gas-only treated control in the leftmost lane were observed after 600 s of treatment for any of the three-jets. Thus, no breakage of the protein backbone was

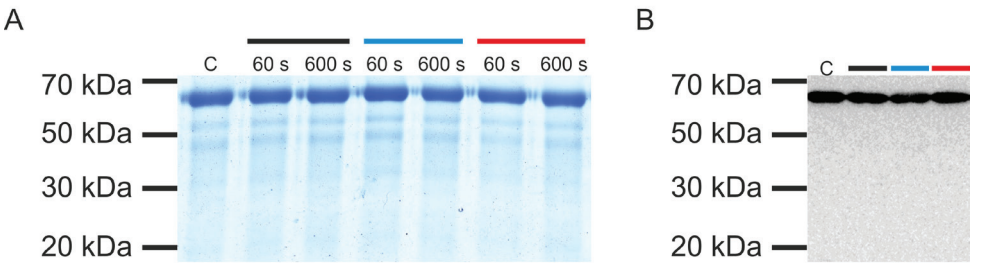

FIG. 2: SDS-PAGE and western blot analysis of plasma-treated BSA samples Samples for SDS-PAGE (A) and western blot analysis (B) were treated with the complete-jet (black), the particle-jet (blue), or the UV-jet (red). Samples for SDS-PAGE were treated for 60 s or 600 s as indicated and western blot samples were treated for 600 s. Controls (C) were treated for 600 s with He/O_2 gas. Experiments were performed twice independently; representative results are shown.

evident. Of the three-jets, the complete-jet features the highest etching rates with 85 nm/min for an a-CH layer.[4] The 10 µg of BSA spotted onto glass with a 3-mm spot diameter formed a protein layer approximately 12.5 µm thick. Assuming equal etching rates as for a-C:H, within 10 min approximately 15% of the sample was removed.

While it is possible that chain breaks occurred, their concentration was below the detection limit of Coomassie R-250 staining (around 30 ng of protein) and low enough not to cause a significant loss in signal intensity at 66 kDa. This result indicates a lack of newly detectable bands and a lack of increased background staining at lower molecular weight and thus insignificant amounts of strand breaks at hotspots or at at random locations throughout the protein. To improve the sensitivity of detection, the experiment was repeated using western blot analysis instead of Coomassie staining. Here, proteins are transferred from the polyacrylamide gel onto a PVDF membrane and subsequently detected by BSA-specific antibodies (Fig. 2B). In addition to the 66-kDa signal from the full-length protein, no additional signals were present, including no increased background at lower molecular weight. Densitometrical analysis of the signals also revealed no significant differences in signal intensities between the controls (average intensity of 74 ± 3 a.u.) and the plasma-treated samples (UV-jet: 73 ± 1 a.u.; particle-jet: 71 ± 2 a.u.; complete-jet 72 ± 3 a.u.). Taken together, we conclude that the plasma treatment does not lead to accumulation of detectable amounts of protein fragments.

B. Raman Spectroscopy of Plasma-Treated BSA Samples

Raman spectroscopy was used to characterize chemical modifications introduced into BSA by X-jet treatment. Samples were treated with the three-jets as described above and Raman spectra acquired. Spectra of samples treated for 600 s were compared to those of controls, which were treated with He/O_2 gas only for 600 s (Fig. 3). Band annotations were taken from the literature.[17–22] Band intensities are discussed relative to the phenylalanine band at 1002 cm^{-1}. In accordance with the experiments described above, Raman spectroscopy confirmed that no amino acid chain breaks occur after treatment with the X-jet or the particle or (V)UV components, as the relative intensities of the amide I bands at approximately 1650 cm^{-1} did not change.

1. Modification of Sulfur-containing Residues

The peaks for the unmodified sulfur residues of methionines at 724 cm^{-1} and cysteines at 734 cm^{-1} were quite weak in untreated controls (Fig. 3A); they only represented one cysteine with a free thiol and four methionine residues of the mature BSA molecule.[23] The relative intensity of the 724 cm^{-1} band decreased slightly after treatment with the complete-jet or the particle-jet, while the intensity of the ν(SO) band at 1043 cm^{-1} slightly increased (Fig. 3B). Taken together, these changes indicate some degree of methionine oxidation. Treatment with the complete-jet and the particle-jet has previously been demonstrated to cause oxidation of the free thiols of cysteines in GapDH.[4] Because no significant change in the cysteine-specific band at 734 cm^{-1} was observed after plasma

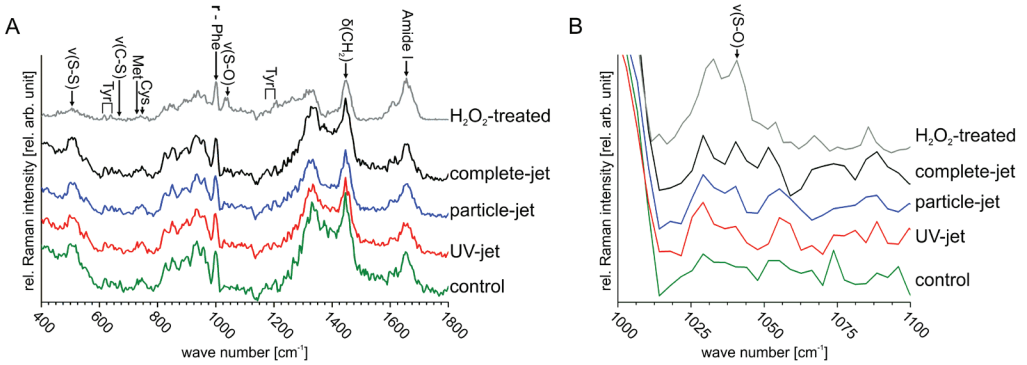

FIG. 3: Raman spectra of X-jet–treated BSA samples Spectra were acquired of samples subjected to treatment with the complete-jet (black), particle-jet (blue), and UV-jet (red). Control spectra (green) were acquired from samples subjected to 600 s of He/O_2 gas treatment, and hydrogen peroxide-treated samples (grey) were used as an additional control to observe sulfur oxidation. Spectra were cascaded for better visibility, and band annotations were taken from the literature.[17–22] A: Relative band intensities are discussed compared to the phenylalanine peak (r) at 1002 cm^{-1}. Cys, Met, Tyr: peaks specific for cysteine, methionine, and tyrosine, respectively. B: Detail of the wavenumber range from 1000 cm^{-1} to 1100 cm^{-1} are shown for better visibility of the ν(SO) band at 1043 cm^{-1}. Experiments were performed three times independently; representative results are shown.

treatment of BSA, we assume that cysteine oxidation does not occur to any major extent in BSA after X-jet treatment. A comparison with hydrogen peroxide–treated samples indicated that sulfur oxidation can occur in principle in the BSA. Hydrogen peroxide is known to cause sulfur oxidations, and for other proteins, a strong increase in the ν(SO) band has already been observed by Raman spectroscopy.[25]

Disulfide bonds, of which BSA has 17, are crucial for protein structure.[24] The relative intensity of the ν(S-S) band at 510 cm^{-1} did not decrease significantly in samples subjected to 600 s of plasma treatment. From these data, it is evident that no significant fraction of disulfide bonds was broken during treatment with the X-jet. In contrast to X-jet treatment, hydrogen peroxide can cleave disulfide bonds, which is indicated by the loss of band intensity around 510 cm^{-1}.

2. Plasma-Induced Tyrosine Modification

Mature BSA contains 20 tyrosines. After treatment with the complete-jet or the particle-jet, a slight increase in intensity can be observed for one of the tyrosine-specific bands at 643 cm^{-1}. A different change occurred after treatment with the UV-jet, namely a decrease in intensity of another tyrosine-specific band at 1209 cm^{-1}. The plasma-emitted particles and photons interacted differently with tyrosine residues of BSA. Photons may have interacted directly with the conjugated double bonds of the tyrosine side chain. Such an interaction can be observed during photon-absorption in the photosynthesis of plants.[26] It

is also known that the (V)UV photons on their way to the sample interact with oxygen in the effluent of the UV-jet, producing protonated water clusters and potentially reactive oxygen species in a photochemical reaction.[15] It is thus also possible that the interaction of photons emitted from the UV-jet with tyrosine were secondary reactions with reactive oxygen species. The nature of the tyrosine modifications introduced by the particles and by the photons emitted from the X-jet remains to be elucidated with complementary methods such as mass spectrometry. In many intracellular eukaryotic proteins, tyrosine phosphorylation plays a key role in modulating protein function.[28] Thus, modification of tyrosines by plasma might be of relevance when treating human cells or tissues.

IV. CONCLUSION

In this study, BSA was used as a model for protein etching by plasma. By analyzing protein size, we showed that protein etching with a modified microscale atmospheric pressure plasma-jet operated with He/O_2 gas does not produce any significant amounts of protein fragmented along the protein backbone. We further demonstrated by Raman spectroscopy that BSA is modified only moderately by treatment with the plasma effluent. We found evidence that some of the methionine residues were oxidized by reactive oxygen species emitted from the complete-jet and the particle-jet, while no significant oxidation of cysteines was detected. Tyrosine residues were subject to modification by plasma-emitted particles as well as emitted photons, leading to different modifications yet to be elucidated. We have confirmed the limited chemical modifications introduced into BSA by plasma treatment, and we conclude, therefore, that BSA is a suitable protein for studies focused on protein etching.

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