NanoSIMS50 analyses of Ar/$^{18}$O$_2$ plasma-treated *Escherichia coli* bacteria

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**Abstract.** Reactive oxygen species (ROS) can be produced by electrical discharges and can be transported in uncharged regions by gas flows, in the so-called afterglows. These species are well known to have bactericidal effects but interaction mechanisms that occur with living micro-organisms remain misunderstood. In order to better understand these interactions, new analysis approaches are necessary. High-lateral-resolution secondary ion mass spectrometry (NanoSIMS) is one of the most promising ways of retrieving additional information on bacteria plasma inactivation mechanisms by combining isotopic imaging of plasma-treated bacteria and the use of $^{18}$O$_2$ as process gas. Indeed, this technology combines a lateral resolution of a few tens of nanometres that is sufficient to image the interior of bacteria, and a high mass resolution allowing detection of isotopes present in low quantities (a few ppm or lower) within the bacteria. The present paper deals with Ar–$^{18}$O$_2$ (2%) plasma treatment, through low-pressure microwave late afterglows, of *Escherichia coli* bacteria and their elemental and isotopic imaging by NanoSIMS. *E. coli* bacteria have been exposed to this reactive medium for varying treatment duration while keeping all other parameters unchanged. Our main goal is to determine whether the quantity of $^{18}$O fixed in treated bacteria and the NanoSIMS50 lateral

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resolution are sufficient to give additional information on E. coli bacteria–plasma interaction.

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1. Introduction

Plasmas are commonly used nowadays for decontamination applications by acting on numerous micro-organisms. Energy dissipation in plasmas leads to the formation of electrical charges, excited neutral species, radicals, photons and gas heating, among others, and all these components can play an important and synergetic role in decontamination processes [1–15]. Nevertheless, interaction mechanisms between plasmas and micro-organisms are still not fully understood [1, 16, 17].

By working under gas flow conditions, it is possible to obtain energetic regions without electrical charges, in the so-called afterglows or post-discharges, and in this way, the reactive medium keeps high reactivity while avoiding ion or electron impact on surfaces. By working with different gas mixtures, e.g. Ar, Ar/O₂ or N₂/O₂, several authors have noticed decontamination and sterilization effects under post-discharge conditions [18]. Correlations between microbial inactivation and UV photons and oxygen atoms have been proposed [5, 9, 10]. UV light can induce DNA damage in the micro-organism and thus it can inactivate it by disabling proper reproduction. Moreover, oxygen atoms produced in such afterglows seem to act as an etching agent by erosion of cellular structures.

This work focuses on the use of the well-known afterglows obtained at reduced pressure with microwaves [19–24]. Plasma production can be carried out using isotopic oxygen (¹⁸O₂), in order to mark the treatment on Escherichia coli bacteria and analyse such labelled microorganisms by NanoSIMS50 technology which allows the elemental mapping of a large variety of small-scale samples (e.g. in material research, geochemistry, cell biology and microbiology).

2. Experimental

In this work, it is assumed that the isotopic exchange (replacement of ¹⁶O by ¹⁸O in ¹⁸O atmospheres) kinetics are far slower (of the order of a few days) than the ones due to the plasma treatment (a few minutes) [25]. This means that the effect of isotopic exchange is always negligible compared with the effect of plasma treatment concerning the quantity of ¹⁸O present in the bacteria cells.

The experimental setup for plasma treatment is depicted in figure 1. A gas mixture composed of Ar and isotopic O₂ (2%) at a total gas flow of 1.02 slm is introduced in the reactor.
Figure 1. Experimental setup.

by adapted mass flow controllers, giving a treatment pressure of 2 mbars. The plasma is created on the left of the 5 mm inner diameter cylindrical quartz tube exposed to 2.45 GHz microwaves by means of a surfaguide wave launcher. The delivered microwave power is 200 W. The species of the discharge are then flowing through the 5 mm inner diameter cylindrical quartz tube, in a zone called the early afterglow, still containing charged species [26]. Then, the early afterglow enters a larger quartz tube (28 mm inner diameter) that is 45 cm from the discharge and is becoming a late afterglow, because no more charged species are present [26]. The afterglow chamber, 60 cm in length, is used to treat biological samples, which are placed 50–60 cm from the discharge.

The distance between the end of the plasma zone and the beginning of the bacteria treatment zone corresponds to 50 cm. The transition from the plasma to the afterglow is characterized by a huge drop of the light emission. The oxygen afterglow is weakly luminescent and it can be seen only in the dark. Its green light is due to the so-called green emission at 557.7 nm arising from the atomic oxygen 1S → 1D transition. This transition is characteristic of the post-discharge where no ions and electrons are present. Then, we can ascertain that the main species in our Ar–O₂ microwave late afterglow, at 2 mbar with a flux of 1 slm of Ar and 20 sccm of isotopic O₂, are singlet O₂ (a¹Δ) and atomic oxygen O, and these species are still present in high quantities (1 × 10¹⁴–1 × 10¹⁵ cm⁻³ with an Ar mixture containing 2% oxygen) [24, 27, 28]. The concentration of these two species is quite constant on a 10 cm length, i.e. on the area of the treated bacteria [26]. No emissions of UV light were observed under such conditions, which induces that reactive oxygen species (ROS) are the main excited species in the afterglow chamber. Unreported optical emission spectroscopy analyses of the afterglow were carried out to confirm these results with an ARC SpectraPro-2500i, equipped with a CCD detector, and a grating of 1200 lines mm⁻¹ blazed at 500 nm. The optical fibre was oriented perpendicularly to the gas flow. Qualitative spectra show weak contamination by nitrogen (estimated to be less than 0.1% in volume).

In these conditions, it is shown that O-containing groups are grafted on the alkane chains during the interaction of the late afterglow with organic compounds [29, 30]. This means that
ROS species are grafted on aliphatic chains (hydroxyl, carboxylic and ketone groups) of lipids or proteins present in bacteria cells during the plasma treatment and then they should be detected by the high-lateral-resolution secondary ion mass spectrometry (NanoSIMS) technique if present in sufficient quantities in the treated bacteria. Then, even if permeation of $^{18}$O$_2$ in bacteria occurs, those oxygen molecules unreacted with the bacteria structure and just absorbed by the cells are easily removed when the biological material is prepared and put under vacuum for characterization. Consequently, the only oxygen species that can be detected within the cell are those that are chemically bonded. ROS such as atomic oxygen are probably more likely to build such bonds, whereas molecular oxygen in ground state gets in and out very easily according to the pressure conditions. Before plasma treatment, $^{18}$O is also present in natural abundance in the molecules constituting the bacteria cells (in hydroxyl, ketone, carboxylic acid and other groups) but not as isolated atomic or molecular oxygen. In order to quantify $^{18}$O coming from plasma from that naturally present in the bacteria, quantities of $^{18}$O in non-plasma-treated but exposed to Ar/$^{18}$O mixture and in plasma-treated bacteria are compared.

In order to observe the influence of heating on the afterglow, temperature was measured in the reactor by using a thermocouple (type K) composed of a tip of 1 mm diameter, which was fixed on the glass slide used to deposit bacteria-containing drops. The glass slide with thermocouple was moved in the treatment zone in order to get a temperature profile along the gas flow direction. Measurements were made after temperature stabilization. It was shown that temperature in the late afterglow is decreasing from about 35 to 28 °C in the direction of the gas flow on the zone where bacteria are located. These average values of temperatures have been obtained for all the experimental conditions used for bacteria treatments.
Our approach to analyse the fixation of $^{18}\text{O}_2$, coming from the plasma, in treated bacteria is presented in figure 2. The model bacterial strain used in this study was E. coli CIP 53.126, a potentially pathogenic Gram-negative bacterium isolated in 1953 from human faeces, and used as a control strain in several standardized microbial testing procedures (DIN 58959-7 [31] and European Pharmacopoeia [32]). The strain CIP 53.126 was cultivated in liquid Tryptone Soya Broth (TSB; Oxoid, Belgium) at 30°C under oxic conditions to an average cellular density of $4.0 \times 10^8$ cells per ml. Ten drops of 10 µl containing this bacteria solution were then deposited on two microscope glass slides (Superfrost, 76 × 26 mm²; Thermo Scientific, Belgium). Then glass slides are introduced in the quartz tube. Ar–$^{18}\text{O}_2$ is introduced first and pressure is lowered mildly in order to avoid the degradation of bacteria, which are sensitive to low pressures. The minimum pressure supported by bacteria during the whole pumping procedure is equal to the treatment pressure (2 mbar), i.e. the pressure was directly decreased from atmospheric pressure to 2 mbars. Then, for the blank samples, Ar–$^{18}\text{O}_2$ is flowing in the reactor without igniting the discharge. For the plasma-treated samples, plasma is ignited just after gas introduction. Treatments were carried out by using the late afterglow effluents generated by an Ar–$^{18}\text{O}_2$ (2%) plasma in the microwave chamber. All parameters were kept constant except the treatment time, which was varied between 1 and 15 min.

3. High-lateral-resolution secondary ion mass spectrometry analyses

SIMS imaging is performed under high vacuum conditions. For this purpose, it is necessary to prepare the samples adequately before analysis. Water present in the structure must be removed but the structure and chemical composition have to be preserved. Protocols used here are similar to protocols used in transmission electron microscopy [33].

To recover the bacteria after plasma treatment, the treated glass slides were rinsed in 1.5 ml physiological water (ultra-pure water with 0.9% NaCl). In order to concentrate the solution, samples were centrifuged (10 min at 8000 g). Then, bacteria were embedded in a 3% agar matrix that was cut into small pieces, a method allowing further treatments without centrifugation. The samples were fixed overnight with 5% glutaraldehyde in phosphate-buffered saline (PBS; 1 mol l$^{-1}$). Glutaraldehyde was removed and bacteria were washed with PBS before being postfixed for 1 h with 1% osmium tetroxide (OsO$_4$) in PBS. After an additional wash with PBS, the samples were dehydrated in several acetone/water solutions with increasing acetone concentration up to 100% and embedded in epoxy resin (epoxy embedding medium). Once the resin is polymerized, 500 nm semi-thin sections were cut by ultramicrotomy (Leica Ultracut UCT) and placed on silicon wafer (Siltronix) as shown in figure 2. The whole sample preparation does not modify the quantity of $^{18}\text{O}$ fixed and reacted on the cell structure because only the liquids (mainly water) contained in the cell structure are removed and replaced by a stable resin during this preparation. We can consider that water or cytoplasm in the cell structure was also reacting with ROS from the afterglow, leading to the formation of some stable compounds containing $^{18}\text{O}$ (e.g. H$_2^{18}\text{O}_2$). By removing these plasma-treated liquids, $^{18}\text{O}$ incorporated into bacteria may decrease slightly, lowering the real effect of plasma.

NanoSIMS imaging was carried out on the Cameca NanoSIMS50 ion microprobe (Cameca, Gennevilliers, France). This SIMS instrument has been developed for high-resolution imaging (with an optimized lateral resolution down to 50 nm) allowing the investigation of subcellular structures and measurement of isotopic composition with high transmission. Cs$^+$ primary ions were generated by a caesium source and accelerated towards the sample surface.
as a tightly focused ion beam with a probe working diameter of approximately 80–100 nm, an intensity of 0.8–1.0 pA and an ion impact energy of 16 keV. The secondary beam was focused and guided through several transfer lenses to the entrance of the mass spectrometer, consisting of a double-focusing filter analyser. The first filter was an electrostatic sector and the second filter was a magnetic sector.

According to the mass-to-charge \((m/z)\) ratio, ions were separated in the magnetic field to a parallel multicollection system allowing the simultaneous detection of up to five masses. In this study, the masses studied were: \(^{16}\text{O}, \ ^{18}\text{O}, \ ^{12}\text{C}^{14}\text{N}, \ ^{31}\text{P}\) and \(^{32}\text{S}\). The SIMS instrument has been carefully tuned to reach a mass resolution of 4500. All images have been acquired in 256 × 256 pixels format with a counting time of 20 ms per pixel.

4. Results and discussion

Different exposure times (1, 5, 10 and 15 min) have been used to treat the samples under the experimental conditions described above. As shown in figure 3, NanoSIMS50 analyses deliver isotopic images of cellular structures for several elements (figures 3(a)–(e)). The first result is that cells can thus be clearly localized. Besides, we measured oxygen fixation pixel by pixel in different intracellular areas for each \textit{E. coli} individually, using NanoSIMS50 to measure the incorporation of oxygen gas enriched in the stable isotope \(^{18}\text{O}\). The \(^{12}\text{C}^{14}\text{N}^-\) ion image as well as carbon, sulfur, phosphorus, fluorine and chlorine ions are typically used to illustrate morphology in organic materials [34–37], and here reveal the bacteria outlines. In these pictures, it is shown that the cell structure (membranes, core) is still observed. It means that the low pressure used (2 mbars) and the afterglow were not aggressive enough to destroy the cell structure. Indeed, the pressure was lowered slowly (a few minutes) and the late afterglow does not contain active etching species (charged species).

The hue–saturation–intensity (HSI) scales represent the measured \(^{18}\text{O}/^{16}\text{O}\) ratio divided by the natural abundance ratio (see figure 3(f)). The scale ranges from blue (the natural value) to magenta (an increase over the natural value by at least the factor indicated on the right of each bar) [38]. The HSI image thus reveals small areas of high excess \(^{18}\text{O}\) located inside the cellular wall as can be seen from figure 3(f). Figure 4 allows us to compare HSI images for the different exposure times: it indicates the increase of isotopic oxygen incorporated into bacteria with treatment time. The blank sample submitted to an Ar–\(^{18}\text{O}_2\) atmosphere for 15 min contains \(^{18}\text{O}\) in natural abundance; that is, for the HSI images, where only the \(^{18}\text{O}\) present in excess of natural abundance is imaged, no \(^{18}\text{O}\) is detected in the blank sample. In contrast, for samples treated for 15 min by the late afterglow, excess of \(^{18}\text{O}\) present in the cell is maximum as shown by higher \(^{18}\text{O}^-\) ion intensities. In order to better understand figure 4, it has to be noted that \textit{E. coli} bacteria are composed of several superposed membranes on the periphery and cytoplasm embedding chromosome, ribosomes and some inclusion bodies in the core, and present a bacillus shape. Then, two cases can be observed after interaction of bacteria with plasma: either reactive plasma species (ROS here) are just present at the periphery (only reaction with membranes) or reactive plasma species are detected on the periphery and inside the bacteria (also reaction with a part of the cytoplasm, chromosomes, ribosomes and inclusion bodies). In our case, the second possibility is evidenced by NanoSIMS. The different bacterial shapes observed with NanoSIMS are due to different orientations of bacteria in the cross-section as explained in figure 2. Bacteria cross-sectioned in the length axis presenting an ‘ellipse shape’ as shown for 10 min of plasma treatment give more information as the whole bacteria structure is
Figure 3. Example of cartography of $^{16}$O, $^{18}$O, $^{12}$C$^{14}$N, $^{31}$P and $^{32}$S for a sample exposed to the Ar/$^{18}$O$_2$ microwave afterglow during 15 min (arrows indicate bacterial position) (a: $^{16}$O, b: $^{18}$O, c: $^{12}$C$^{14}$N, d: $^{31}$P, e: $^{32}$S, f: HSI image ratio $^{18}$O/$^{16}$O). HSI image of the $^{18}$O/$^{16}$O ratio derived from ($^{16}$O) and ($^{18}$O). The colours correspond to the excess $^{18}$O derived from the measured $^{18}$O$^{-}$/^{16}$O$^{-}$ isotope ratios, expressed as a percentage of the $^{18}$O excess in the bacteria, which is a measure of oxygen renewal; values range from natural abundance to higher accumulation (magenta).

easily observed. It can be deduced that ROS produced in the afterglow interact with the organic matter of cells and are present in sufficiently high quantities to be detected by NanoSIMS50. Moreover, the lateral resolution of our method is sufficient to evidence the penetration and fixation of ROS, both on the membrane and inside the cell.

To compare each plasma treatment, guided by the HSI image, we have calculated the values of the $^{18}$O/$^{16}$O ratio and the percentage of penetration of $^{18}$O for individual bacteria. An increase of isotopic oxygen incorporated into the structure as a function of the exposure time has been
observed (figure 5). Saturation after 5 min of exposure seems to indicate that the action of ROS produced in the afterglow is limited to the first minutes of treatment. It is thought that the saturation value in fixed \( ^{18} \text{O} \) corresponds to the maximum number of sites available for fixation of ROS in the cell structure. After 5 min, equilibrium is reached between the destruction of sites containing \( ^{18} \text{O} \) groups and the creation of new reactive sites for \( ^{18} \text{O} \), as observed in the degradation of organic molecules by similar late afterglows [29, 30]. It may be possible that the other saturation value is obtained for longer treatment times if further large degradation of cell structure occurred. The reason why the saturation value is only twice the natural ratio is that the number of fixation sites for \( ^{18} \text{O} \) is quite low in the probed area (cross-section only 500 nm thick) compared with the total volume of the treated bacteria.
These studies show a very interesting result concerning the interaction mechanisms between micro-organisms such as the *E. coli* bacterium and the excited species produced in afterglows. Indeed, as the gas used for plasma production is labelled by isotopic oxygen, these results ensure that ROS considerably act on the cells. These results complete the analysis made by several authors [5, 9, 10] showing ROS-induced degradation of molecular structures to form volatile products (CO₂ and H₂O). ROS may either induce a non-ionic etching process or cross the membrane and consequently penetrate the cells in order to inactivate them.

Indeed, recent results [29, 30, 39, 40] obtained on heavy alkanes and fatty acids with the same experimental setup and in the same experimental conditions shed light on the effect of diffusion processes of the ground state and the singlet state of O₂ on the etching mechanism of these compounds in liquid phase. Because of their mobility in liquids, radical chains created by hydrogen abstraction after reaction with oxygen atoms can react with O₂ or O₂(a¹Δg). Peroxide compounds are thus created. As they are labile, they reorganize by breaking the closest C–C bond. Light fragments produced by this mechanism are gaseous and they are removed from the liquid, etching the material. Heavy fragments, with various functional groups such as alcohol, ketones or acids, are detected after treatment in the residual material. Such a mechanism could explain the presence of ¹⁸O in the core of the bacterium.

5. Conclusions

By using afterglows in Ar/¹⁸O₂ gas composition, it is possible to remove electrical charges and to considerably reduce heating of the excited gas and UV irradiation. ROS as atomic oxygen and singlet O₂(a¹Δ) are thus mainly produced in addition to Ar-excited species, and
their interactions with micro-organisms such as the model bacterium *E. coli* have been observed by using NanoSIMS50 analyses. This technique is shown to be a very promising technique for the analysis of interaction mechanisms between plasma-generated reactive species and living organisms because of its high lateral resolution, high sensitivity and mass resolution. Degradation of *E. coli* bacteria by ROS has been evidenced. Finally, the preparation of the treated bacteria, the tuning of the NanoSIMS parameters and the methods used to treat the NanoSIMS images have to be well optimized. In future, different sample preparations will be compared in order to optimize the NanoSIMS analyses of treated bacteria.

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