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BACTERIAL INHIBITION EFFECT OF PLASMA ACTIVATED WATER

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Abstract. The properties of the liquids change at the interaction with electrical discharges. This offers the so activated liquids a variety of possible applications in the biomedical field. The paper investigates the bacterial inhibition effects of plasma activated water on *Staphylococcus aureus*. Three discharges in He, Ar, respectively air were used for water activation. The effect of the treatment time and liquid storage time were both considered. The plasma activated water proved to be efficient for bacterial inhibition, holding its properties for at least 7 days. Hydrogen peroxide was revealed to be the main inhibition agent generated at the plasma liquid-interaction.

Key words: nonthermal plasma, plasma activated water, bacterial inhibition.

1. INTRODUCTION

Biomedical applications of non-thermal plasmas in contact with liquids represent a recent interdisciplinary research field located at the intersection between physics, chemistry, material science, medicine and biology. Research studies show new initiatives to develop and improve the biomedical applications of atmospheric pressure plasmas [1]. Non-thermal plasmas can be used directly, on or in contact with the living tissue for applications in disinfection and sterilization, dermatology, cancer treatment, dentistry and endoscopy. Discharges are also involved in indirect medical applications as treatments are applied on different surfaces, materials or medical devices in order to optimize various medical procedures [2, 3]. Therefore, non-thermal plasma treatments were successfully applied for biodecontamination in hospital hygiene, medical equipment sterilization, for antifungal treatment, dental care, skin disease or chronic wounds healing [1, 2, 4].

Plasma sources can be classified depending on the type of feed gas, the power transferred to plasma, the waveform and the frequency of the electric applied field, the electrodes geometry and the gas temperature [5]. When used for biomedical applications, the discharge setups must respect constrains referring to general electrical safety regulations, ultraviolet photons generation and reactive

species production [6]. Several types of plasma discharges are suitable for biomedical applications, but the most often used are the dielectric barrier discharges (DBD), plasma jets and μ-jets and corona discharges [7, 8, 9]. Their advantages are the low gas temperature (close to room temperature) [10], the non-aggressive character at the interaction with materials and the small sizes of the discharges which allow their application on local surfaces [2, 10]. Moreover, plasma discharges can be applied on living tissues in order to heal diseases without affecting the healthy skin [11].

Plasmas in contact with liquids initiate various chemical and physical processes at the interface region between the plasma and the liquid [12, 13]. Depending on the type of the discharge, on the plasma energy and on the chemical composition of the liquid, a number of primary species are generated in the gaseous phase, in the liquid phase or at the plasma-liquid interface [12]. Thereby, reactive oxygen species (ROS) like hydroxyl radical (·OH), atomic oxygen (O), ozone (O_3) and hydrogen peroxide (H_2O_2) have an important role in the process of water activation by plasma treatment [14, 15]. Moreover, reactive nitrogen species (RNS) like nitrites (NO_2^-), nitrates (NO_3^-) and peroxynitrites also influence the characteristics of the plasma treated liquids [16]. The non-thermal plasmas change the physical and chemical proprieties of the liquid with which they interact and produce molecules dissociation, electrons and ions, increase the pH value and can produce UV radiation [17]. One of the most important mechanisms of producing reactive species is the water dissociation process that results in the formation of positive hydrogen ions (H^+) and hydroxyl radicals in the liquid phase.

Research studies show that some bacteria do not react to classical antibiotics treatments because they have developed resistance genes [18]. Also, it was shown that UV radiation and reactive species can produce harmful effects when interacting with biomolecules, especially with DNA, causing strand breakages and possible damages that can lead to mutation and death [2, 18, 19, 20]. Cold atmospheric pressure plasmas can generate reactive atoms and molecules within or in close proximity to the living tissues [21] that can change the chemical processes of the cellular membrane or the permeability of the cell [22, 23]. Scientific studies show that while in wet environments or water based-liquids bacteria show high degrees of development, when using plasma activated water (PAW) on the same cultures of bacteria the living and growing conditions of the microorganisms are limited [10, 24–26].

The current work shows the inhibitory effects produced by plasma activated water on *Staphylococcus aureus*. The bacteria was selected because it is one of the most common infectious agents as it causes numerous skin and respiratory infections, food poisoning and it is very dangerous in hospital hygiene [27, 28]. Moreover, *Staphylococcus aureus* is an unpretentious bacteria that can grow even in very simple habitats [29]. The water is activated by exposure to two discharges: to a plasma micro-jet in He or Ar or to a micro-arc in air. The PAW is further

incubated with the microorganisms and its inhibition effect on the bacteria is assessed. The treatment time influence, the importance of the discharge and the storage potential of the PAW are all investigated.

2. EXPERIMENTAL DETAILS

2.1. WATER ACTIVATION

The experimental setup used for water activation consists of two μ-jet discharges in Ar or He and a μ-arc discharge in air which are schematically represented in Fig. 1 and was previously described in [30]. Two different powered electrodes are employed. The first electrode is used to generate the μ-jet Ar or He discharge and it is a capillary metallic tube (syringe needle, 0.6 mm inner diameter) with a free end which is placed 4 mm above the water surface. The gas (He or Ar) passes through the electrode with a flow rate of 0.3 l/min. The second electrode is used to generate the μ-arc discharge in air and it is a Kanthal A-1 (Fe 71.02%, Al 5.8%, Cr 22%, Mn 0.4%, Si 0.7%, C 0.08%) sharp wire (1 mm diameter and 6 mm length) fixed into a brass holder. The second electrode is also placed 4 mm above the water. Both discharges have common characteristics like the electrical signal applied (in terms of frequency of the electric field applied and the power transferred to the plasma –16 W) and physical parameters like the distance between the electrode and liquid surface. For both discharges, each electrode is powered by high frequency sinusoidal voltage (1.7 kV, 10.2 MHz) generated with a laboratory made "free-running" oscillator. The second electrode of the discharges has a floating potential and is represented by the treated water. The volume of the treated water samples was 35 ml.

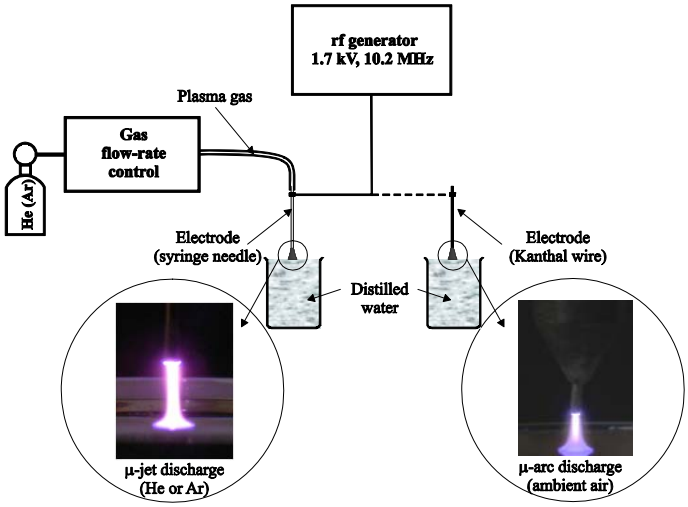

Fig. 1 – Schematic representation of the plasma generator.

2.2. BACTERIAL INHIBITION

The inhibition effect of the PAW samples was investigated by using *Staphylococcus aureus (S. aureus)* bacteria as test microorganisms. An overnight bacteria culture that was cultivated in nutrient broth was further incubated at 37°C for 24 h with the PAW samples. The volume ratio of the nutrient broth and the active agent was varied between 10:0 and 0:10. The inhibition effect of the PAW was investigated by determining the optical density of the bacterial suspensions at 620 nm using a Jasco V-530 UV-VIS spectrophotometer. Each value obtained represents the average of 5 different measurements. Control samples of bacteria incubated with similar volume ratios of nutrient broth and distilled water and samples without dilutions of the nutrient broth were also investigated.

To understand better the effects of the species generated by the plasma that influence the bacterial inhibition process, the *S. aureus* cultures were also exposed to synthetic solutions containing hydrogen peroxide and a mixture of hydrogen peroxide and nitric acid. The solutions were prepared so that the H_2O_2 concentration and the final pH value were identical to the values obtained when the water was activated for 50 min with the He μ-jet.

The effects of several discharge parameters like discharge gas, treatment time and storage time for 7 days are all investigated.

3. RESULTS

3.1. VOLUME RATIO DEPENDENCE

Bacterial cultures were incubated for 24 hours with the PAW samples. *Staphylococcus aureus* was used as test microorganism and water activated by exposure to the helium micro-jet for 50 minutes was the test inhibition agent. Different volume ratios of growth medium: PAW were investigated. Considering that the addition of water to the growth medium can influence the growth process of the bacteria by direct dilution of the nutrient broth and by changes induced to the osmotic pressure in the cells [31], control samples in which distilled water was added to the growth medium in similar volume ratios as the PAW were also investigated. The sole effect of the water activation process was estimated using the following formula:

$$Bacterial\ inhibition = \frac{O.D._{dw} - O.D._{PAW}}{O.D._{nb}} \quad (1)$$

where *O.D.* represents the optical density value at 620 nm, *dw* stands for distilled water, *PAW* for the plasma activated water and *nb* to the control sample for which the nutrient broth was not diluted. The results are shown in Fig. 2.

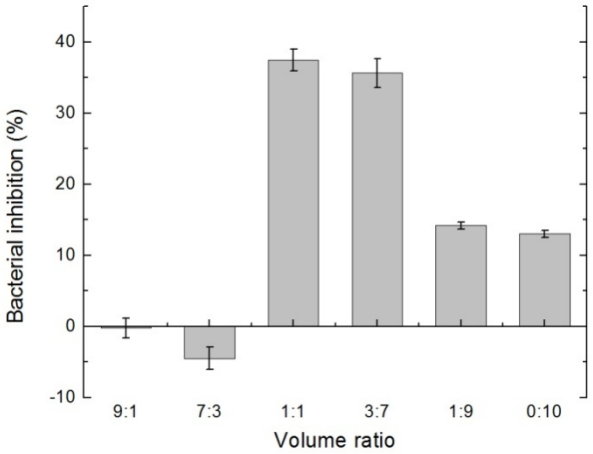

Fig. 2 – Effect of nutrient broth: inhibition agent volume ratio on the bacterial inhibition process. PAW obtained after exposure to the He μ-jet for 50 minutes was used.

It can be observed that for the volume ratios in which the growth medium is more prominent, the inhibition effect of the PAW is not relevant. Moreover, it appears that the PAW aids the development of the microorganisms. In the case in which the inhibitory agent exceeds the nutrient broth in volume, the effect of the plasma treatment loses its relevance due to the fact that the bacteria are inhibited by the important changes of nutrient concentration and osmotic properties of the liquid [32]. Therefore, the difference between the inhibition effect of the PAW and of the distilled water is not very prominent. Figure 2 also shows that the volume ratio in which the effect of the water activation process is evidenced the most is 1:1. For this ratio, the concentration of inhibitory species is sufficient for bacterial inhibition and the water quantity added to the growth medium is not too big to overwhelm their effect. Consequently, the following experiments were performed using only the 1:1 volume ratio.

3.2. TREATMENT TIME DEPENDENCE

The effect of the treatment time on the interaction between PAW and *S. aureus* was investigated. The results obtained using the water activated with helium μ-jet are shown in Fig. 3. It can be observed that for low treatment times, the PAW appears to be beneficial for the microorganisms and to stimulate their growth. By increasing the treatment time to 30 minutes, the PAW becomes harmful for the bacteria and a mild inhibition effect of the PAW is highlighted. A further increase of the treatment time results in a stronger inhibition degree of the bacteria. The results are aligned with the trends presented in the literature as the increase of the treatment time generates more active species in the PAW and offers the PAW a stronger antibacterial effect.

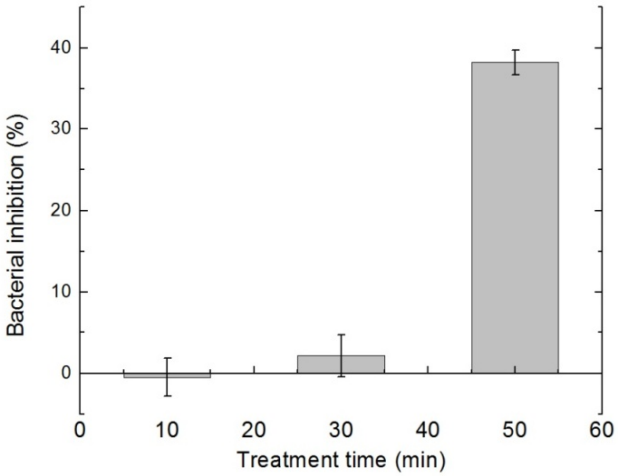

Fig. 3 – Bacterial inhibition effect of PAW – treatment time dependence.
PAW obtained after exposure to the He μ-jet was used.

3.3. DISCHARGE TYPE INFLUENCE

The effect of the discharge type used to activate the PAW for identical treatment times was also investigated. Figure 4 shows that the use of the different discharges offers different results for each discharge. It can be seen that the air discharge offers the lowest inhibition rate while the Ar discharge shows the more intense effect.

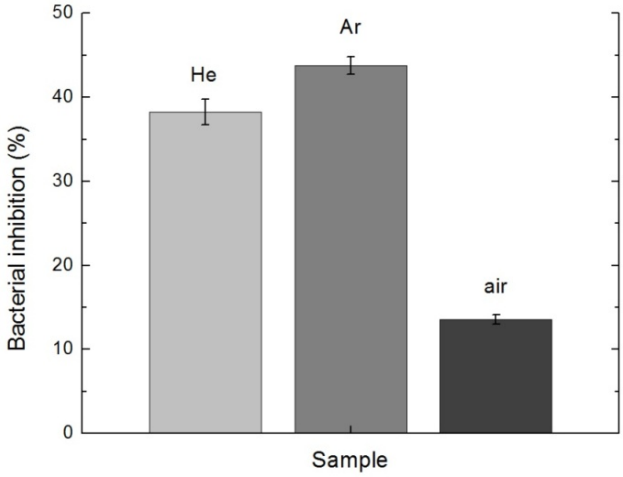

Fig. 4 – Bacterial inhibition effect of PAW – discharge type influence.
PAW obtained after exposure to the plasmas for 50 minutes was used.

Considering the properties of the PAW summarized in Table 1, it can be observed that the Ar discharge creates the highest concentration of hydrogen peroxide in the corresponding PAW sample, followed by the helium and air discharges, which is similar to the inhibition effect on the *S. aureus* samples. Furthermore, by increasing the treatment time of the PAW samples, the concentration of hydrogen peroxide is also increased.

Table 1

Characteristics of the PAW

Treatment time [min]	pH			H_2O_2 [mM]		
	He	Ar	air	He	Ar	air
10	2.9	3.01	2.56	0.26	0.55	0.22
30	2.13	2.42	2.03	0.61	0.91	0.36
50	1.79	2.19	1.72	0.91	1.19	0.52

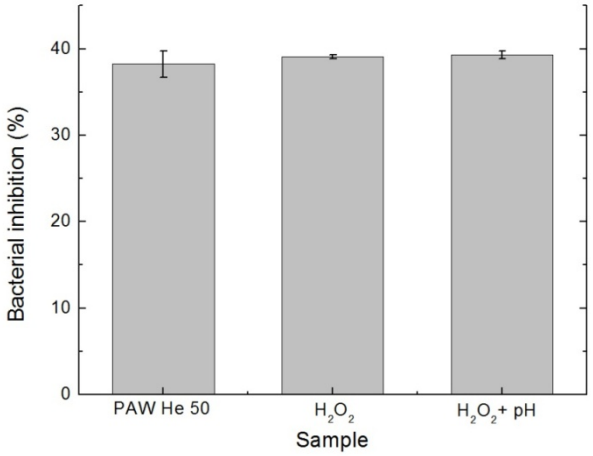

Fig. 5 – Comparison of the inhibition effect of PAW activated by exposure to the He μ-jet and synthetic solutions with properties similar to the PAW.

A reasonable assumption is that the hydrogen peroxide is strongly related to the inhibition rate of the bacteria as a result of PAW addition to the growth medium. To confirm this supposition, the effect of two synthetic solutions on *S. aureus* was examined. The first solution is a H_2O_2 solution with a concentration of 0.91 mM and the second is a H_2O_2 solution with a concentration of 0.91 mM and a pH value of 1.79 obtained by addition of nitric acid to the solution. The properties of the synthetic solutions were chosen to be similar to the PAW obtained by exposure to the He discharge for 50 minutes. Figure 5 shows a comparison of the effects had by the PAW and the synthetic solutions. It can be seen that the hydrogen peroxide solution has a similar inhibition effect as the PAW sample.

Moreover, the low pH value brings no further advantage to the synthetic solution and the inhibition effect of the second synthetic solution is equal to the one of the first synthetic solution and to the PAW sample, confirming the hypothesis that H_2O_2 is the main inhibition agent that is formed in the PAW as a result of exposure to plasma.

3.4. STORAGE POTENTIAL

The storage potential of the PAW was investigated. Before the addition to the bacteria, the PAW samples were stored under room temperature, dark conditions, in closed plastic containers. Figure 6 shows the results. It can be seen that even if the ratio slightly varies, the inhibition effect of the PAW is still considerable.

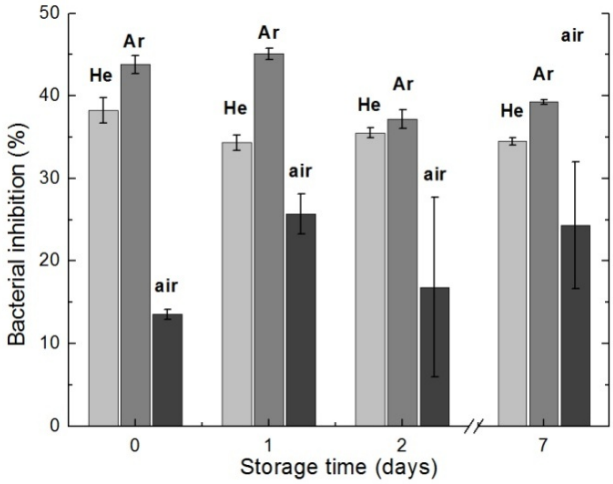

Fig. 6 – Bacterial inhibition effect of PAW - storage time dependence.

4. DISCUSSION

The above results show that a small quantity of PAW active species added to bacteria growth medium does not affect the evolution of the microorganisms or, contrary to our wish, aid their growth process. This trend is evident both in the case of volume ratio and treatment time dependencies. However, a higher quantity of active species formed in the activated water results in a considerable inhibition effect of *S. aureus*. This is most evident when the treatment time is increased to 50 minutes and the discharge type is changed.

The hydrogen peroxide emerged as the main active species that is formed at the plasma-water interaction in terms of *S. aureus* inhibition. Comparing our results with the literature it can be seen that the opinions regarding the main factors of

bacterial inhibition at plasma-water interaction are divided. Laurita *et al.* [33] linked the antimicrobial activity of the water to the pH of the solution and to the formation of peroxynitrite in the PAW solutions. Kojitari *et al.* [34] also identified the HONOO radical as main antibacterial species, mentioning that H_2O_2, HNO_3 and HNO_2 in the amounts generated in their samples can not be responsible for bacterial inactivation. Traylor *et al.* [35] considered that a joint effect of H_2O_2 and nitrite is responsible for the inhibition for short PAW-bacteria interaction time. In [36] and [37] the authors consider the ROS species as main inhibitory agent. Oehmigen *et al.* [25] have shown that synthetic solutions of nitrate, nitrite, hydrogen peroxide or HCl (pH = 3) or combinations of these species do not show similar antimicrobial activity as plasma treated NaCl solutions and that a longer exposure time of the bacteria to the active species results in a more remarkable inhibition effect. Other works identified several active agents for the bacterial inactivation process and suggest that a synergistic effect of pH value, oxygen and nitrogen species [16, 24], is the complete explanation for the inactivation of bacteria at plasma-liquid interaction. Considering all these opinions, we could suppose that there are several mechanisms that contribute to the inhibition process. The pH value, the reactive oxygen and the reactive nitrogen species can all be responsible for antibacterial effects. Depending on the species generated by the plasmas, the final concentrations of RONS in the PAW or the time the activated water is in contact with the microorganisms, one of the agents mentioned above could become dominant, similar to our work in which H_2O_2 proved to be the main agent.

The storage potential of the PAW proved to be in our case at least 7 days. This contrasts with the results presented in [33], where the lifetime of the water was considered 25 minutes. In [25], the authors showed that the inhibition effect of the PAW is reduced remarkably in the first 30 minutes after plasma exposure. Shen *et al.* [26] have investigated the antimicrobial effect of PAW stored at different temperatures and concluded that the PAW stored at room temperature loses its antimicrobial efficiency in a few days after the plasma exposure and that the storage under freezing conditions at –80 deg. C proved to increase the storage potential of the PAW. Traylor *et al.* [35] have shown that when a PAW sample interacts with a bacterial culture for short time intervals (15 min), the inhibition efficiency shortly drops with the increase of the storage time, but when the PAW interacts with the microorganisms for 3 hours, the storage potential of the activated water can reach up to 7 days. However, if the results concerning the over 21 days time stability of the PAW properties, previously published in [30], are considered and the main antimicrobial agent of the PAW is identified as the H_2O_2, also regarding the 24 hour PAW incubation time with the microorganisms, the long storage potential shown by our PAW samples is fully explained.

It might be argued that the PAW treatment is not necessary to obtain solutions with antibacterial properties and that a direct plasma treatment or a solution obtained *via* a chemical route is simpler and more economically efficient. It is a fair statement.

However, in a world in which the medical applications of non-thermal plasmas are growing in momentum, and most of the body tissues have high water concentrations, the side effects of the species generated at the plasma liquid must be fully understood. Under such considerations, the inhibition activity of the PAW might prove itself useful as a secondary effect of body plasma exposure, especially if its effect lasts for days.

5. CONCLUSION

The distilled water activated by exposure to non-thermal plasmas showed important inhibitory effect on *S. aureus.* The treatment time of the liquid samples is associated with different concentrations of active species that are formed in the water and with varied effects on the bacterial cultures. While a small treatment time is associated to an almost inexistent effect on the microorganisms, 50 minutes of treatment result in considerable inhibition effects. The argon discharge proved to generate the most antimicrobial PAW, and the main inhibition agent was identified to be the hydrogen peroxide, while the low pH value did not substantially impact the process. The inhibition potential of the PAW samples proved to be very stable, for 7 days after plasma exposure the changes were minor, even if the water samples were stored at room temperature.

The plasma activated liquids represent a very complex medium as it contains several chemically active agents. When it takes contact with bacterial cells, several antimicrobial mechanisms can be stimulated inhibiting the development of the microorganisms. The processes are not fully understood yet, but each work is a step forward for more clarity.

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