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The effect of non-thermal plasma Jet on bacterial biofilms and plasmid DNA

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ABSTRACT

The effect of non-thermal discharge plasma (DBD) on the inhibition of both gram-positive and gram-negative biofilms bacteria was examined at different plasma exposure times and gas flow. This effect induces damage to aqueous plasmid DNA. It reveals inactivation in bacterial biofilms for both types of bacteria with an increase in time of direct exposure to plasma and an increase in gas flow. The presence of positive bacteria outweighs the negative bacteria in susceptibility to inhibition. The result fractions of the DNA are indicate the whole DNA double-strand breaks and were determined using agarose gel electrophoresis. The damage level induced in the plasmid DNA is also enhanced with increased plasma irradiation time.

KEYWORDS: Plasma DBD; Non-thermal plasma; Bacteria biofilm inhibition; Plasmid DNA.

INTRODUCTION

Plasma represents a state of matter in which gas molecules are separated into positive and negative ions and electrons. Recently, the atmospheric cold plasma (ACP) at room temperature such as dielectric barrier discharge (DBD), was used due to the advantages offered in terms of cost, ease of processing, and environmental compliance. This type of plasma has wide uses and applications in medicine, sterilization, and heat-sensitive materials. The issue of sterilization is still a major topic, sterilization, and killing of microorganisms. Plasma is a fast and efficient process Non-toxic, low-risk to workers, operators, and patients. The efficacy of Non-thermal plasma for microbial inhibition has been demonstrated [1, 2, 3]. Potential applications of ACP now include environmental, food processing, clinical, and health care. As a result of lipid oxidation [4] resulting from plasma generation of different chemically active agents that affect surface microbes such as the surfaces of medical devices and vital tissues [5, 6, 7]. Different responses to inhibition have been observed in studies of plasma sterilization. Negative bacteria were found to have greater resistance than positive bacteria [8]. While the opposite was found in other studies [6, 7], and in studies that did not show significant differences in the effect of plasma treatment between Gram-positive and Gram-negative bacteria [9, 10]. Higher plasma voltages and longer treatment times are more effective in inhibiting it [1, 11, 12, 2]. The direct plasma exposure method can cause stress to cells, and the active ions can directly affect cells exposed to plasma [13, 14]. The reactive types generated may include reactive oxygen types (ROS), reactive nitrogen types (RNS), ultraviolet radiation, active ions, and charged molecules. ROS may play the most important role in microbial inactivation [2]. Hydrogen peroxide (ROS) considered to have a strong oxidizing effect on microbes as well as ozone, all of which can be produced using plasma. [13] In addition, RNS significantly improves the chemical and microscopic properties of infected vital tissues. It can be easily developed by controlling the gas flow on the treated surface [15, 16]. The type and extent of the reactive types generated can influence the deactivation mechanism. This range of reactive types may vary

depending on the composition of the inert gas used. In this paper, the deactivation effect was studied by non-thermal plasma with a DBD discharge type using helium gas. The study of the effect on the biological materials that were used, which included the two types of positive and negative bacteria, and was applied to the plasmid DNA.

Plasma experimental setup

An atmospheric pressure plasma jet (APPJ) system was used in the current study as shown in Figure 1. The APPJ is generated inside a dielectric tube, with an inner diameter of 0.5 mm and an outer diameter of 0.65 cm, and two insulated copper electrodes are used as an electrode. The electrodes are connected to a high voltage power supply and inert gas helium was flows inside the tube to generate the plasma jet.

Figure 1. Plasma system.

Preparation of bacterial biofilms

Biofilms details

For two types of Gram-negative bacteria and two types of Gram-positive bacteria (*E. Coli*, *Klebsiella*, *Lactococcus*, *Streptomyces*) grown under suitable growth conditions. The biofilm was prepared by several steps based on the method [17, 18, 19].

1. A colony of 3-4 fresh bacterial cultures was suspended in glass tubes containing (1.5 ml) of Tryptone Soy Broth medium (TSB), at a concentration (0.1 - 0.08) that was determined using a photometer with a wavelength of 620 nm.
2. Four columns with four rows were selected from a plate (96) holes, meaning that a column was left between each two columns and a row between each two rows.
3. TSB medium without bacterial suspension was distributed over the pits of the first column at a rate of 250 µl (Figure 2).
4. The solution containing the bacterial suspension was distributed over the pits of the remaining five columns also at a rate of 250 µl and the plate was tightly wrapped to prevent any contamination. Two more columns in a second panel, leaving empty gaps between them, supplemented the columns. Then the plate was incubated for 48 hours.
5. Remove the solution from all the holes, then wash the holes with normal saline solution one time, to get rid of non-adherent cells. Then place the dish in the incubator for 10 minutes for drying purpose.

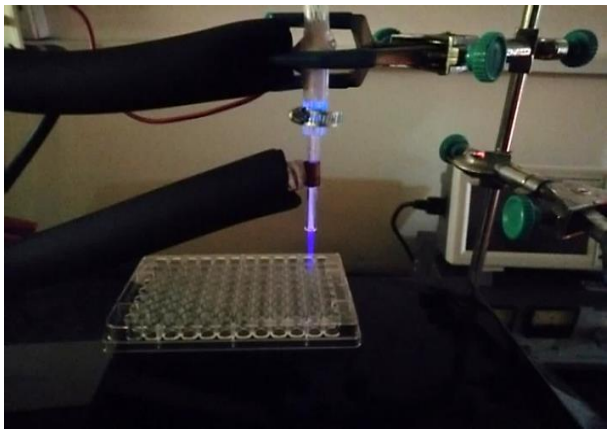

Figure 2. The mean in the pits of the plate.

Plasma biofilm treatment

The biofilm samples were exposed to plasma according to the following steps:

1. The plate in which the biofilms were formed was exposed to the plasma, while the first and second columns were left. These columns are considered (negative control) and (positive control), respectively.
2. A specific distance is placed between the plate and the plasma column.
3. The third column was exposed for a period of (one minute) and the four coefficients of one column were replicates for the same time period.
4. The fourth column containing biofilms was exposed for a longer duration (3 minutes) and so on with the other two columns (5 minutes) and (10 minutes) respectively to obtain four columns exposed to plasma with four replicates for each column. For the two additional columns, the first column was exposed only to gas without

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applied voltage with its repeaters, and the last column was exposed only to high voltage without gas for the two columns' specific time period of 3 min.
5. After the plasma exposure ended, all pits were washed with saline and for the purpose of biofilm stabilization; the plate was placed in an oven at 60 °C for 30 min.
6. To detect biofilm density, the pits were stained with 0.1% safranin dye for 15 minutes and then washed with distilled water. The dye was extracted from etching the plate with ethanol (99.9) for a period of time (5 min), after which the absorbance was measured using an ELISA device at a wavelength of 490 nm.
7. These steps were done for three different gas flows.

Preparation of plasmid DNA

Plasmid DNA (PBR322), a common cloning vector for Escherichia coli, prepared from Thermo Scientific, was used at a concentration of 0.5 µg/µl. The plasmid was prepared in several steps according to [20]:

1. The first amount that is considered control and the four quantities are exposed to the plasma at different times (0.5, 1, 2, 4) minutes.
2. Prepare 1.5% agarose gel, with distilled water, with gentle stirring and heating, then add 5 µl Red Safe and keep stirring.
3. The electrophoresis optical laboratory is equipped with a tray for loading the transfer device, with dimensions of 10 cm by 15 cm. The edges were tightly wrapped with masking tape and a special drilling comb was fixed at the tip of the gel.
4. The solution was added and left to cool and solidify to obtain a solid gel
5. The comb and adhesive tape were removed and the plate was fixed in the relay trough with the drill side moved to the negative side of the electrodes and immersed in 1X TBE solution (TBE 10X is prepared by mixing 4 g of NaoH with 20 g of boric acid in a beaker until dissolved at low temperature He adds 800 ml of distilled water at pH 8.5).
6. 8 µL of the plasmid sample was added with 2 µL of the loading solution into the pits with caution. 10 µl of a 100-base pair ladder was added into a slot on one end of the gel.
7. The electrodes of the relay were connected to a 5V/cm power source of generation, the relay was carried out towards the positive pole for about 3 hours, and the dye was observed until it reached the end of the generation, the relay process was stopped.
8. Then we lifted the plate from the sink, and the final image of the gel sample packages was revealed using an ultraviolet device.

RESULTS AND DISCUSSION

Bacterial biofilms

Table 1 shows the absorbance analysis and arithmetic mean and percentage of decrease in the membranes for both gram-positive and gram-negative biofilm bacteria at different plasma exposure times and gas flow after DBD plasma treatment.

The tabular results and graphs that show the percentages of bacterial inhibition appear:

that is a clear decrease in the proportion of biofilms of bacterial species commonly used using non-thermal atmospheric pressure plasma, gradually with increasing exposure time. This finding has been supported by studies [21, 22].

Table 1. the percentage of inhibition in the biofilm of bacteria

Gas flow (L/ min)	Exposure times	E. Coli			Klebsiella			Lactococcus			Streptomyces		
		Arithmetic mean	Standard deviation	Percentage change (%)	Arithmetic mean	Standard deviation	percentage change (%)	Arithmetic mean	Standard deviation	Percentage change (%)	Arithmetic mean	Standard deviation	Percentage change (%)
1	C.P	0.450	0.021		0.386	0.023		0.335	0.020		0.288	0.014	
	1 min	0.408	0.015	9.41	0.358	0.030	7.42	0.283	0.022	15.62	0.209	0.019	27.51
	3 min	0.376	0.026	16.52	0.324	0.037	16.13	0.251	0.017	25.17	0.196	0.017	31.91
	5 min	0.355	0.017	21.04	0.308	0.036	20.19	0.236	0.022	29.45	0.185	0.008	35.84
	10 min	0.331	0.032	26.52	0.281	0.021	27.35	0.214	0.015	36.12	0.176	0.012	38.96
2	C.P	0.498	0.069		0.416	0.042		0.326	0.026		0.345	0.033	
	1 min	0.450	0.053	9.51	0.343	0.021	17.55	0.269	0.034	17.47	0.270	0.026	21.74
	3 min	0.390	0.022	21.63	0.314	0.031	24.60	0.212	0.018	35.14	0.238	0.020	30.92
	5 min	0.364	0.041	26.93	0.308	0.097	25.96	0.189	0.026	42.19	0.216	0.019	37.39
	10 min	0.339	0.035	31.88	0.284	0.036	31.81	0.150	0.012	54.03	0.203	0.022	41.06
3	C.P	0.447	0.043		0.349	0.036		0.342	0.023		0.336	0.031	
	1 min	0.356	0.029	20.37	0.295	0.016	15.49	0.220	0.021	35.61	0.276	0.020	17.87
	3 min	0.291	0.020	34.85	0.239	0.038	31.45	0.179	0.011	47.61	0.199	0.018	40.71
	5 min	0.260	0.019	41.79	0.225	0.034	35.47	0.127	0.023	62.73	0.163	0.007	51.34
	10 min	0.247	0.025	44.63	0.192	0.021	45.03	0.108	0.006	68.29	0.144	0.017	57.10

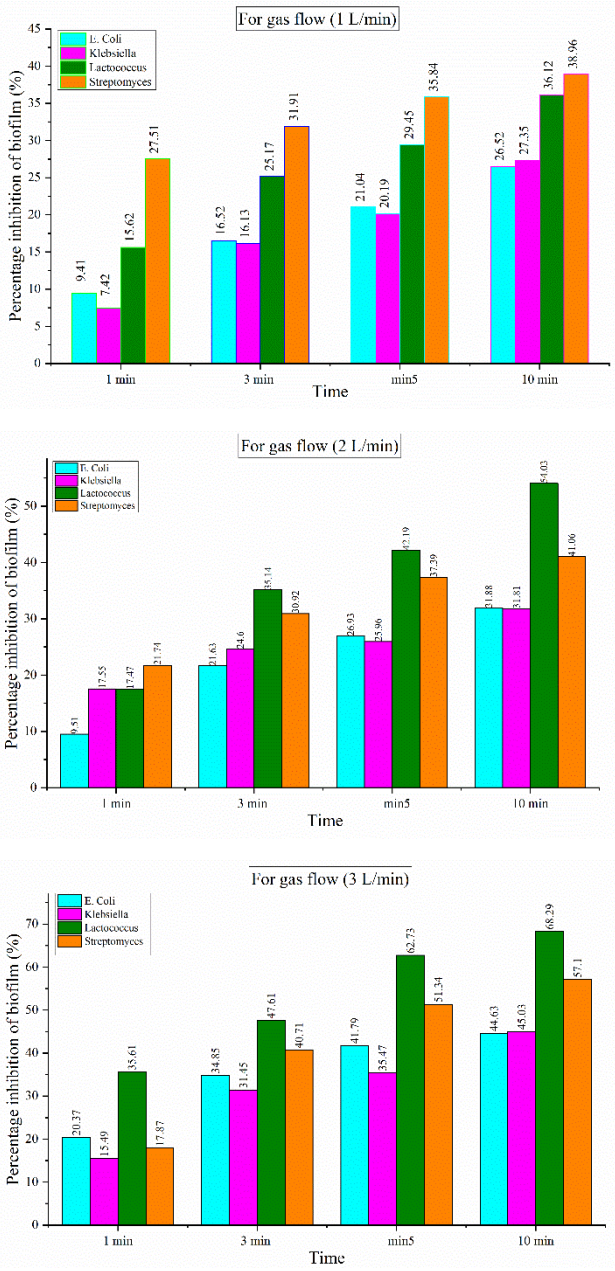

Figure 3. The percentages of the biofilm inhibition at 1, 3, 5 and 10 min exposure times for all gas flow.

From figure 3 it is noticed a clear increase in the ratio with the increase of the helium gas flow, which was clearly shown in the exposure time of 3 minutes, which was proven by some studies including ref. [22].

There is one difference in the rate of deficiency for all types of bacterial biofilms and it varies according to the type of each used bacteria from the other. However, the percentage of deficiency in the positive bacteria (*Lactococcus*, *Streptomyces*) was greater than in the negative bacteria (*E. Coli*, *Klebsiella*), which was evident in the periods of high exposure and high flow, especially in the case of flow (3 L/min), At the exposure time of 5 minutes, the highest growth rate of *lactobacillus* positive bacteria was 62.73, and the lowest growth rate was in *Klebsiella* negative bacteria, which reached (35.47). As for the exposure time of 10 minutes, the highest percentage was also for lactobacilli (68.29), and the lowest for *E. Coli*-negative bacteria (44.63). The reasons for this result is that the negative bacteria are more resistant to antibiotics than the positive bacteria due to the physiological difference in the composition of the outer envelope of both the negative and positive bacteria. Which has been proven by other studies including [8, 23].

As for the transactions that were treatment by gas only and the other by voltages only, through work and measurement, was noticed no change was observed in their value from the value before exposure, and this indicates that no effect occurred. [24, 25]. So the deficiency that occurs in the bacterial biofilms is due to the effect of the active elements resulting from the generation of plasma, which are represented by oxygen, nitrogen and their oxides and other elements, and Which has been proven by other studies [26, 27].

In more detail, we can say that the main active reactive elements available in plasma, which include charged particles, active oxygen species (ROS-O, OX, OH) and active nitrogen types (RNS-N, NOX) as well as ultraviolet radiation, all of these elements together are believed to be Responsible for the effectiveness of antimicrobials. This effect on the cellular level by the resulting active elements due to the generation of plasma is due to the oxidation process. Thus, the reactive elements (RNS, ROS) have strong oxidative effects on the external cellular structures. They act on lipid oxidation, DNA damage, protein programming and modification, and cell death in microorganisms [24, 28]. As for the charged particles, their role lies in the dismantling and tearing of the outer part of the cell membrane of bacteria. As the electrostatic force that results from the accumulation of charged particles on the outer cell membrane overcomes the bonding force and the tensile force that binds it and works to shredded it [25].

Thus, the increase in the continuous flow of gas for longer periods of time resulted in a continuous

increase in the production of the active agents ROS and NOS, and this in turn led to the accumulation of these gases on the sample to be treated. Thus, a decrease in biofilms resulted. Biofilms treatment is considered one of the important treatments because bacterial biofilms are generally more resistant to antibiotics than swimming bacteria, as they resist external factors and harsh environmental conditions [29].

In addition to the presence of influencing factors resulting from electrical discharge on microorganisms the non-thermal plasma with the insulating barrier technology is considered one of the techniques that is distinguished from other techniques in that it works to inactivate bacteria safely, because it does not cause any harm to living tissues, due to the low temperature of the jet. The plasma does not rise much above room temperature, but it should be noted that the decrease in biofilms also depends on the density of bacterial cells or bacterial biofilms, in addition to exposure time and exposure dose [2].

Plasmid DNA

After exposing four samples of plasmid DNA to non-thermal plasma (DBD) at atmospheric pressure in four different time periods. The result of the effect was detected using the UV device after the samples were migrated through the generation of agarose using the electrophoresis device.

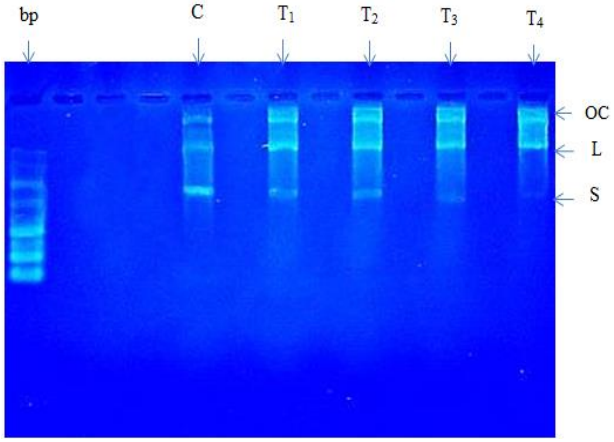

Figure 4. The image of plasmid sample packages in the agarose gel.

Figure 4 shows an image of an agarose gel containing plasmid sample bundles migrated using electrophoresis after exposure to plasma at different time intervals.

While:
bp: weight index, which is 100 bp
C: sample without treatment (reference)
T1: First transaction (0.5) minutes
T2: Second transaction (1) min
T3: First transaction (2) minutes
T4: First transaction (4) minutes

Figure 4 shows the migration of plasmid samples during the process of electrophoresis from the drilling site where the samples were injected near the negative electrode and directed towards the positive electrode. The second group is called Linearized and the third group is Supercoiled, respectively. Initially, the ratio of the Supercoiled group is the highest among the linear and circular shapes or groups.

By observing the shape of the samples in the gel through the UV apparatus, it was found that the plasmid was gradually destroyed with increasing exposure time [30]. Where the shape of (sopercoil) began to decline clearly with the increase in the time of exposure to plasma, which seemed clear to its permanent demise. The circular and linear variety has increased in relative value. This situation can be explained by the fact that the circular formation can be increased and produced due to the process of DNA strand breakage (single strand break), and it is also possible to degrade and produce the linear formation by the two processes, which are one or two DNA strand breaks (SSB) or (DSB), this is because the SC ratio is high in the first exposure times and because the rate of SSB is an order of magnitude higher than that of DSB. Thus, an increase in the production rate of the circular group OC was obtained from the two processes, and this led to an abundance (OC). Thus, there will be a clear decrease in (SC). There is a persistent increase in LIN with continued exposure to plasma due to its formation by SC and OC groups [31]. These results are consistent with other studies that have shown the same damage and similar behavior of the plasmid, with differences in the periods of exposure to plasma with longer periods. So that it turned into a DNA, fingerprint due to prolonged exposure to plasma. Which represents part of the damaged DNA plasmid, and transformed it into polynucleotide fractions with a number of lengths [32]. The main factor for this change in genetic material is due to the increased concentration of reactive types resulting from ionization and gas electric discharge from plasma generation, as I mentioned earlier, having a strong effect on nucleic acids and organisms. Thus, when the process is continued or the plasma exposure

period is increased, it leads to significant DNA damage.

The study of the effect of plasma on DNA is one of the most important applications of biomolecules because it plays an important role in the function of storing genes and genetic information, which will be passed on to future generations. Therefore, DNA is an important target of plasma radiation (Arjunan K. et al, 2015).

CUNCLUSION

In this study, a DBD- plasma jet, operating at 9 kV atmospheric pressure, Power 5.67 W and frequency of 100 kHz, showed a pronounced effect on bacterial biofilms, where exposure to plasma biofilms inhibited bacterial biofilms and reduced the strength of the bacterial biofilms. This effect increases with increasing exposure time and increasing gas flow. In addition to studying its effect on plasmid DNA, its exposure to plasma resulted in a decrease in supercoil formation and an increase in linear and circular morphology with increasing exposure time. Thus, when exposure to plasma continues, plasmid DNA will be destroyed. The discharge non-thermal plasma jet (DBD) under atmospheric pressure is low and close to room temperature, and thus is of great use in the medical field.

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