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Measurement of hydrogen peroxide concentrations in plasma activated media

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Abstract: Plasma activated media (PAM) is very effective in killing cancer cells. One of the key reactive species found in PAM is hydrogen peroxide which is known to have biological implications. Here we report on the measurements of H_2O_2 concentrations using fluorescence. The results indicate that the concentration of H_2O_2 reaches a saturation value in less than 1 min. The onset of saturation time gets shorter for higher voltages.

Keywords: hydrogen peroxide, low temperature plasma, plasma medicine, cancer, RONS

1. Introduction

Low temperature plasma (LTP) can either be applied directly on cells and tissues to cause biological effects, or it can be used to "activate" a medium which is subsequently applied to a biological system (cells, tissues, organs). Recently several investigators have shown that plasma activated media (PAM) is effective in killing cancer cells [1, 2]. Low temperature plasma generated in oxygen or air containing environments produces various chemically reactive species such as OH, O_2^-, NO, and O that are known to have biological implications [3, 4]. However, these species have short lifetimes and do not penetrate deep in liquid media. On the other hand, they can interact with the liquid to generate relatively stable long lived species inside the volume of the liquid. One such reactive species is hydrogen peroxide (H_2O_2). Hydrogen peroxide is known to cause various oxidizing reactions in biological cells, including the peroxidation of lipids and DNA damage. In this paper we report on the use of Amplex red hydrogen peroxide/peroxidase assay kit to measure hydrogen peroxide concentrations created by the plasma pencil used on Minimum Essential Medium, MEM. Voltage pulses with amplitudes ranging from 5 kV to 9 kV at constant pulse width were used to drive the plasma pencil. MEM was exposed to the plasma for various exposure times ranging from 0 to 4 minutes. After application of the Amplex red assay a microplate reader was used to measure fluorescence. The excitation wavelength was 544 nm while the fluorescence emission was detected at a wavelength of 590 nm. The results indicate that the concentration of hydrogen peroxide generated in the MEM reaches a saturation value (around 28 μM) in less than 1 min and the onset of saturation gets shorter for higher voltages.

2. Materials and Methods

An Amplex red hydrogen peroxide assay kit was used in this study. This kit contains Amplex red reagent, dimethylsulfoxide (DMSO), 5 X reaction buffer, horseradish peroxidase, and hydrogen peroxide. This kit must be stored at -20 °C and protected from light for optimal use. The Amplex red reagent is one of the most commonly used to measure concentrations of hydrogen peroxide. Some other substrates that could be used for similar purposes are homovanillic acid and diacetyldichloro-fluorescein [5]. With any of these fluorescent products, increases in the amount of hydrogen peroxide react with the fluorescent product to form an increase in fluorescence. When Amplex red is used, the Amplex red is oxidized by the hydrogen peroxide when horseradish peroxide (HRP) is also present. This combination creates resorufin, a highly colored compound that can be detected using different methods. One is colorimetric which can detect resorufin using absorption at 570 nm. Another is by fluorescence which can detect resorufin using emission around 585 nm and excitation around 570 nm. In this work, to record the fluorescent light intensity a microplate reader (BMG Labtech FLUOstar) was used with an excitation wavelength of 544 nm. The fluorescence was collected at a wavelength of 590 nm. In this assay the intensity of the fluorescence is typically proportional to the concentration of hydrogen peroxide.

The plasma pencil was used in this study to generate the low temperature plasma. The plasma pencil is a made of a hollow dielectric tube with a diameter of 2.5 cm [6, [7]. It has two disc electrodes about the same diameter as the tube that are inserted in the tube with a 0.5 cm separation and are each composed of a thin copper ring attached to a centrally perforated dielectric disc. Fig. 1 below is a schematic of the device.

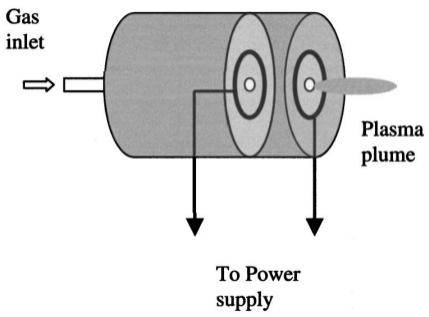

Fig. 1. Schematic of the plasma pencil.

To ignite the plasma, a high voltage of 1 - 10 kV was used in the form of a repetitive, microseconds-wide pulses and applied between the two electrodes. The operating gas such as helium with flow rates in the 4 - 6 slm range can be used. When the discharge is ignited, it launches a plasma plume that can reach up to 5 cm into the ambient room air.

For each experimental sample, 500 μL of a liquid medium of MEM was pipetted into a 24 well plate. Helium gas at a constant flow rate of 5 slm was used. The bottom of the plate of samples was placed at a constant distance of 2.5 cm from the nozzle of the plasma pencil. The pulse generator was kept at a constant frequency of 5 kHz and constant pulse width of 1 μs. For the experimental results presented in this paper, we used three pulse amplitudes of 5, 7, 9 kV and we tested exposure times at 15 seconds, 30 seconds, 1 minute, 2 minutes, and 4 minutes. A schematic of the experimental setup is shown in figure 2 below.

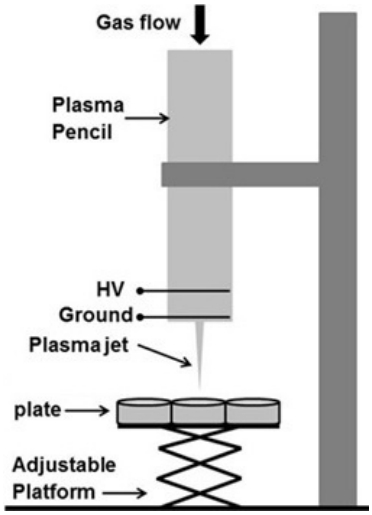

Fig. 2. Experimental setup.

Following an intricate protocol (not described here) recommended by the kit manufacturer to prepare the stock solutions, 50 μL of the standard curves and experimental samples were pipetted into individual wells of a 96 well plate. The contents of Amplex red vial were dissolved in DMSO to create a 10mM Amplex red stock solution. Next, 25 μL of Amplex red stock solution, 50 μL of 10 U/mL HRP stock solution, and 2.425 mL of 1X reaction buffer were mixed to create 2.5 mL of the working solution. Then 50 μL of this working solution was added to each microplate well containing the controls and the experimental samples. This was then incubated at room temperature for 30 minutes, while being protected from light. Lastly, the 96 well plate containing the standards and the experimental samples was introduced into a microplate reader to collect the fluorescence emission. As mentioned above, an excitation wavelength of 544 nm and an emission wavelength of 590 nm were selected.

3. Results and Discussions

The first task consisted of constructing the standard curve. This standard curve was made up of diluted 3% hydrogen peroxide with MEM into concentrations of 20 μM, 15 μM, 10 μM, 5 μM, and 2.5 μM. A sample of MEM was also used with no hydrogen peroxide added and no exposure to plasma. This was used for our 0 μM. Fig. 3 is a plot of the standard curve.

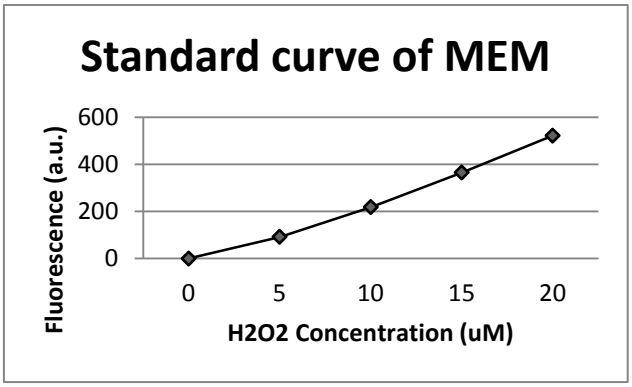

Fig. 3. Standard curve relating the fluorescence intensity to the concentration of H_2O_2.

Fig. 4, Table 1, and Fig. 5 show the measured fluorescence and concentrations of H_2O_2 for three voltage amplitudes at five different plasma exposure times.

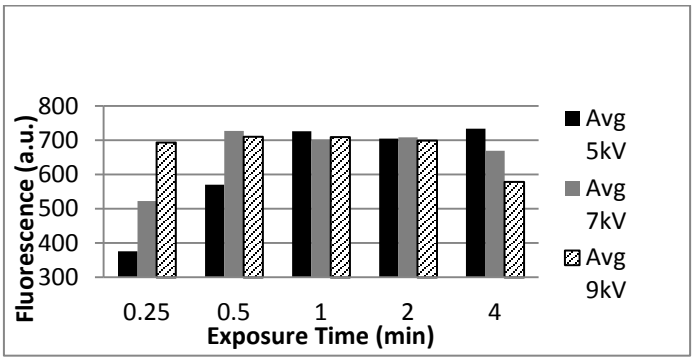

Fig. 4. Measured fluorescence values.

Table 1. H_2O_2 average concentrations (in μM) for different voltages and for various exposure times.

Time (min)	5kV	7kV	9kV
0.25	15.17	20.79	27.30
0.5	22.59	28.56	27.93
1	28.54	27.57	27.91
2	27.73	27.86	27.50
4	28.83	26.35	22.92

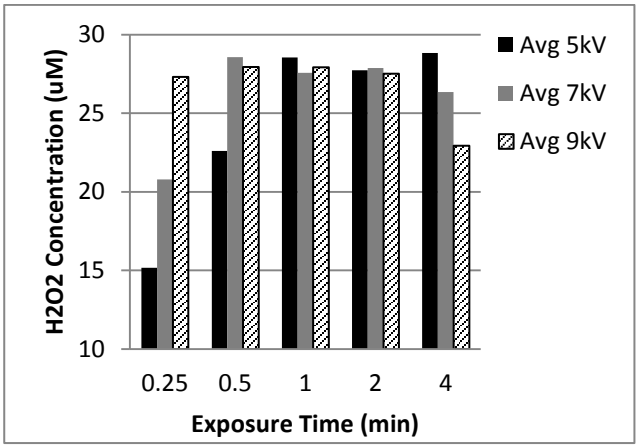

Fig. 5. H_2O_2 average concentrations for different voltages and for various exposure times.

Based on the above results we can see that at 5 kV, the fluorescence and H_2O_2 concentration increase until around 1 minute exposure time. At 1 minute, the fluorescence values and H_2O_2 concentration stop increasing and remain around the same value (28 μM), for the rest of the times. At 7 kV, the fluorescence and H_2O_2 concentration exposure increases only until 0.5 minute exposure time. After 0.5 minute exposure time, the fluorescence remains around the value with a slight decrease at 4 minutes exposure time. The H_2O_2 concentration stays around a value of 28 μM and then decreases at 4 minutes. At 9 kV, the fluorescence reaches a saturation value at 0.25 minutes and remains around this level until 4 minutes exposure time. The corresponding H_2O_2 concentration is around 28 μM. At 4 minutes, the concentration seems to drop.

From these results some observations can be made. At an exposure time of 0.25 minutes, the hydrogen peroxide concentration for 5 kV is the smallest and the largest concentration is for 9 kV. This is a result that was expected, as with larger applied voltages, the hydrogen peroxide concentrations would increase. At an exposure time of 0.5 minutes, the concentration for 5 kV is smaller than those at 7 kV and 9 kV, which further supports this as well. Once the hydrogen peroxide concentrations level off around 28 μM, it appears that it has reached saturation. There were no more reactants to bond together and form the hydrogen peroxide. This saturation point seems to be reached quicker, the higher the voltage applied. For 5 kV, the saturation was not reached until 1 minute exposure time. At 7 kV, saturation was reached already at 0.5 minutes, and at 9 kV, saturation was reached even quicker, already at 0.25 minutes. The drop of fluorescence and hydrogen peroxide concentration at 4 minutes could be error from the measurements or possibly another influence. This could be studied further in future experiments.

4. Conclusions

When applied to a liquid medium, the plasma pencil produces a large amount of hydrogen peroxide in a short amount of time. After a certain length of time, the concentrations of hydrogen peroxide no longer increase, at which time the saturation point is reached. The minimum voltage used in this experiment, 5 kV, was able to reach the saturation point by 1 minute. Also, the larger the voltage used, the faster this point of saturation was reached. At 9 kV, the saturation point was already reached by 0.25 minutes. The Amplex red assay kit worked very well in identifying the concentrations of hydrogen peroxide produced by the plasma pencil. This kit would also work to measure the concentrations of hydrogen peroxide in cells.

5. References

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