

## Application of Atmospheric Pressure Argon Plasma Jet (APAPJ) in Biomedical Science and Engineering

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### Article history:

Submission December 2019

Revised February 2020

Accepted April 2020

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### ABSTRACT

In this paper, we demonstrated the application of atmospheric pressure argon plasma jet (APAPJ) in biomedical science such as cancer therapy and biomedical engineering such as surface modification of polystyrene Petri dishes for animal cell culture. We observed that APAPJ, when exposed to breast cancer cell line (MDA-MB-231) for 60 seconds at a distance of ~ 2.5 cm, reduced the cancer cells by 57%. This reduction in cancer cell concentration demonstrated the cancer-killing property of APAPJ. Further optimization of APAPJ's various operational parameters and in-depth biochemical study is required to figure out the mechanism responsible for killing cancer cells. Also, we investigated cell adhesion and proliferation due to APAPJ treatment by Crystal Violet (CV) staining and MTT Assay. We observed that APAPJ treatment of polystyrene Petri dishes for 60 - 240 s at a distance of ~ 3.5 cm exhibited enhancement in adhesion of breast cancer cell line (MDA-MB-231). We also demonstrated the increase in hydrophilicity by a reduction in contact angle to  $30.3^{\circ} \pm 3$  after 240 seconds of APAPJ treatment of polystyrene samples. The hydrophilicity of the substrate has proven to enhance cell adhesion. Consequently, APAPJ could be employed as a simple, inexpensive and environmentally friendly method for surface modification of polystyrene plates for animal cell culture.

*Keywords:* APAPJ, breast cancer cells, MTT assay, contact angle

### Introduction

Plasma is an ionized state of matter consisting of electrons, ions, neutrals, radicals, excited particles, and UV-radiation. Recent progress in atmospheric plasmas has led to the creation of cold plasmas with ion temperature close to room temperature which is employed to treat various biological samples such as DNA [1], microorganisms [2] and cancer cells [3]. This biomedical application of cold atmospheric plasma has led to the emergence of the new promising field in biomedicine termed as 'Plasma Medicine'.

Cancer is one of the dreadful diseases prevailing in this planet caused by different agents that attacks various body organs and parts such as colon, breast, brain, pancreas, and leukemia. In cur-

rent cancer therapy, three major interventions are performed step by step or/and in combination to treat cancer viz surgery, chemotherapy and radiation therapy. But these interventions lack precision and accuracy to distinguish between normal and cancer cells. Mainly in terms of selectivity and efficacy, plasma is emerging as a promising therapy to treat cancer cells with minimal side effects. Reactive oxygen species (ROS) and reactive nitrogen species (NOS) which are produced during plasma generation are thought to be responsible for selective killing of cancer cells with possible induction of the apoptosis pathway [4]. Oxidative stress which is generated by these reactive plasma species is hypothesized to kill cancer cells with no

### How to cite:

Gurung JP, Shrestha R, Subedi DP, Shrestha BG (2020) Application of Atmospheric Pressure Argon Plasma Jet (APAPJ) in Biomedical Science and Engineering. *Journal of Tropical Life Science* 10 (2): 149 – 154. doi: 10.11594/jtls.10.02.07.

significant inflammatory/hypersensitive reaction in normal cells.

Various adhesion proteins such as collagen and fibronectin in addition to physical methods such as gamma-ray irradiation is used to enhance cell adhesion in polystyrene substrate [5]. These approaches are expensive, harmful to the operators due to radioactivity and cause pollution when discharged into the environment after use. Polystyrene is widely used as a substrate for monolayer growth in animal cell culture. As manufactured, polystyrene is hydrophobic in nature and is treated with corona discharge,  $\gamma$ -radiation or chemicals to produce charged and wettable surfaces. An increase in hydrophilicity due to surface modification enhances the adhesion and spreading of cells in a substrate. During surface modification by plasma discharges, the generation of certain functional groups like  $-\text{COOH}$ ,  $-\text{C}=\text{O}$  and  $\text{OH}$  groups occurs in the treated surface [6]. This modification is significantly favorable for cells to adhere and proliferate. Atmospheric pressure plasma discharges such as plasma jet are also used to treat polystyrene substrate, enhances cell adhesion by a surface modification to more hydrophilic nature which is quantified by contact angle measurement [7]. In this paper, we have demonstrated the application of atmospheric pressure argon plasma jet (APAPJ) in cancer therapy and surface modification of polystyrene Petri dishes for animal cell culture.

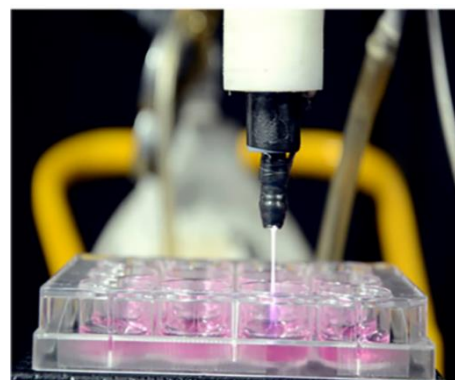
## Material and Methods

### Experimental setup

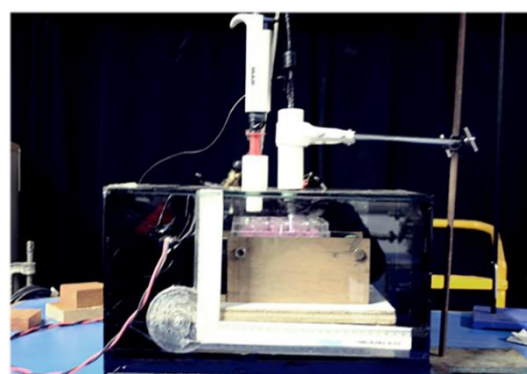
APAPJ was generated to treat cancer cell-lines cultured in 12-well culture plates (Corning Inc.) (Figure 1A). To establish sterile conditions for treatment, a chamber was constructed termed as 'Portable Treatment Box' (PTB) (Figure 1B). PTB is a glass box that consists of an input chamber facilitated with a rack to insert cell culture plate, door for fumigation and treatment chamber with pencil shaped APAPJ. It is also facilitated with a rotating belt to roll well culture plates for treatment. Besides, polystyrene Petri dishes were also treated with APAPJ to study its effect on cell adhesion and proliferation.

### Fumigation

PTB was sterilized by fumigation before every treatment and air contamination is monitored by



(a)



(b)

Figure 1. APAPJ treating cancer cells in 12 well culture plates (a) and Portable Treatment Box (PTB) (b).

the settle plate method. During a fumigation, a product of the reaction between formaldehyde solution and potassium permanganate generates fumes that sterilize the box. As fumes are also toxic to biological cells including our sample of cancer cells, treatment is done only after the fumes are removed by using the box only after about 5-6 days. During the settle plate method [8], open workspace and PTB treatment chamber were exposed with agar plates for 30 and 60 min to observe if PTB is sterilized enough to experiment.

### Polystyrene sample preparation

Polystyrene Petri dishes were cut into 30 mm by 15 mm and cleaned using an ultrasonic cleaner for 10 minutes in distilled water after a few minutes of alcohol washing for plasma jet treatment.

### Cell-line and cell culture

Breast cancer cell-line (MDA-MB-231) cell-line was revived from  $-80^{\circ}\text{C}$  cryopreserved vials. The cell lines were cultured in T-75 flask in

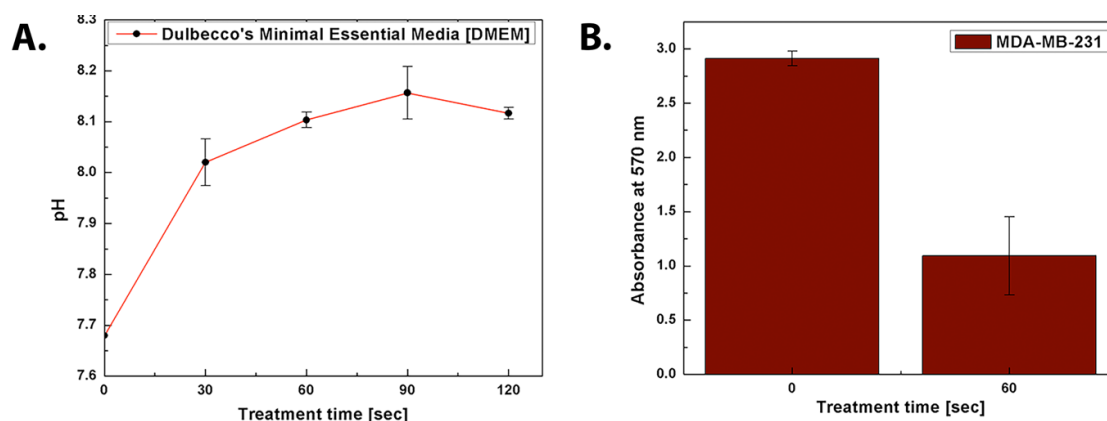


Figure 2. pH value of DMEM media in various plasma treatment time (0 - 120 seconds) (A). MTT assay of MDA-MB-231 breast cancer cell-line treated by APAPJ. Mean  $\pm$  SE plot of absorbance at 570 nm vs treatment time; 0 and 60 seconds after 48 hours of incubation (B).

DMEM cell culture media at 37°C, 5% CO<sub>2</sub>, and 95% humidity in a CO<sub>2</sub> incubator. After 48 hours of incubation, cells were detached from the surface using trypsin and media was added to stop the activity of trypsin. Hemocytometer was used to calculate cell concentration before loading into 12 well tissue culture plates. A monolayer of breast cancer cell MDA-MB-231 was grown on 12 well flat bottom tissue culture plates (Corning Inc.) and transferred to PTB for plasma treatment.

#### Plasma treatment

APAPJ was operated at a high voltage of 7 kV, high frequency of 27 kHz and argon gas flow rate of 2 L/min. MDA-MB-231 cell lines were treated with APAPJ for 30 and 60 sec. The distance between APAPJ nozzle and suspension surface was adjusted at 2.5 cm. PTB is facilitated with the pipette that is used to drain out the media from a well plate (Figure 1B). Consequently, only a thin layer of media is left which prevents dehydration of cells. Eventually, cancer cells are treated at 3.5 cm from the nozzle of APPAJ. Finally, the drained-out media is loaded back in the well plates using the same pipette for MTT assay. In other experiments, polystyrene samples are treated with plasma jet and eventually seeded with MDA-MB-231 breast cancer cell lines to test cell adhesion and proliferation.

Polystyrene samples and petri dishes were treated with APAPJ for four different time lengths (60, 120, 180, 240 s, and Argon gas only for 240 s). APAPJ was operated at a high voltage of 7 kV, a high frequency of 27 kHz and an argon gas flow rate of 2 L/min. The distance between APAPJ nozzle

and the polymer sample was adjusted at 3.5 cm. Treated polystyrene samples (30 mm by 15 mm) were transported for contact angle measurement whereas treated polystyrene Petri dishes were sealed with parafilm and transported to animal cell culture laboratory. In animal cell culture laboratory, cancer cells were loaded only after irradiation by UV for 15-20 minutes to reduce contamination.

#### Temperature and pH

pH and temperature of the media were measured using a pH meter and IR-thermometer to observe any media modification due to plasma treatment.

#### Contact angle measurement

Contact angle with water was measured in treated samples by a sessile drop technique using Ramehart Goniometer [9].

#### CV staining

Crystal Violet (CV) staining, is a blue-colored dye used in staining cells for observation and can also be used for quantification of cell number. It is a type of nuclear staining which stains the cell nucleus which is live or viable. The photograph is captured by CCD camera in a phase-contrast microscope for different treatment times to observe the cell attachment in treated polystyrene Petri dishes.

#### MTT Assay

MTT is a dye consisting of Thiazolyl Blue Tetrazolium Bromide. Cancer cells were incubated

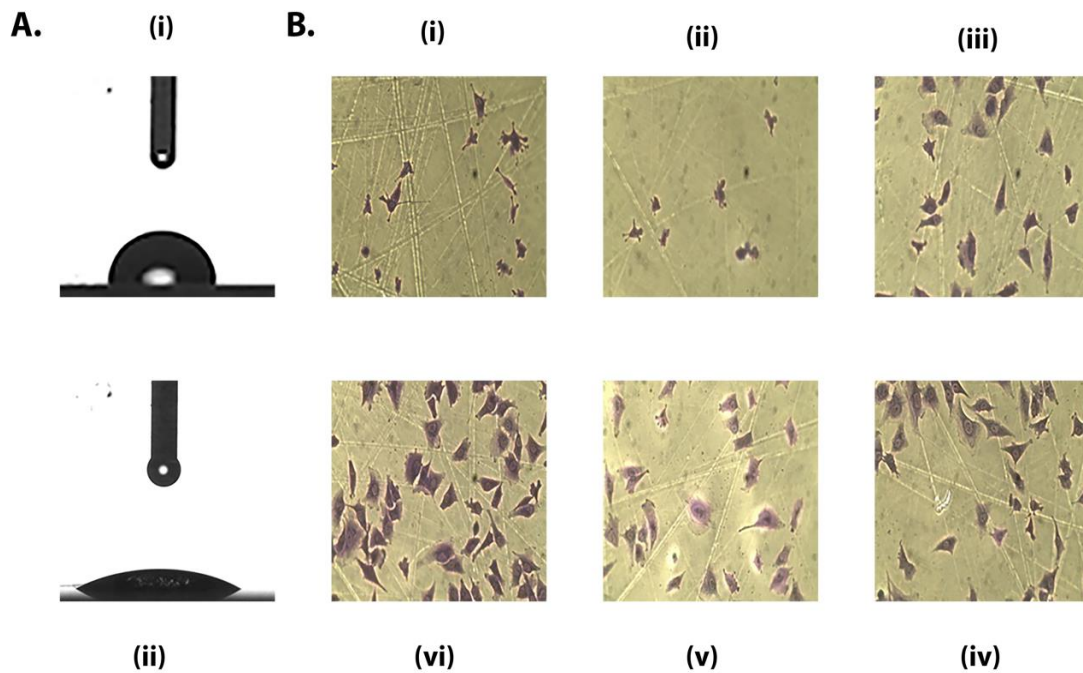


Figure 3. Contact angle images of APAPJ treated polystyrene samples for 0 minutes (i) and 4 minutes (ii) (A). CV staining of MDA-MB-231 breast cancer cell-lines, grown after treating polystyrene petri dish by APAPJ (40× magnification) for 0 to 240 seconds (i-vi) (B).

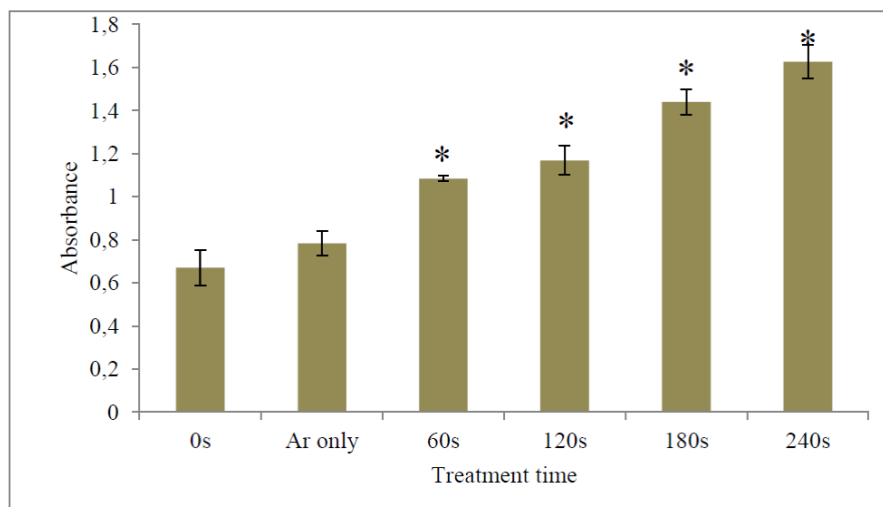


Figure 4. MTT assay of MDA-MB-231 breast cancer cell-line incubated 48 hours in APAPJ-treated polystyrene Petri dishes. Mean  $\pm$  SE plot of absorbance at 570 nm versus treatment time of 0 - 240 seconds. (\* indicates p-value  $\leq$  0.05).

for 4 hours at 37°C in treated cell culture flask with control. After removal of the supernatant and washing with PBS, the isopropanol solution was added. The results were read at 570 nm in an ELISA reader. MTT dye forms purple formazan crystals in metabolically active cells. These crystals are dissolved by isopropanol and absorbance is measured at 570 nm taking reference at 635 nm.

Cell viability was calculated as a function of absorbance obtained from ELISA Reader at 540 nm taking reference at 635 nm.

## Results and Discussions

### Anti-cancer activity of plasma jet

Due to the non-thermal properties of the plasma jet, there was no increment in temperature

Table 1. Contact angle measurement on polystyrene samples treated by APAPJ in different treatment time ranging from 60 – 240 seconds.

Treatment time (seconds)	60	120	180	240
Contact angle [θ] with water (°)	35.2±2	33.1±1	29.6±5	30.3°±3

of media whereas a slight increase of pH by 1 log difference was observed in DMEM media (Figure 2). This slight change in temperature and pH does not affect the cells significantly. The settle plate method confirmed that PTB is free of air-contamination as agar plates placed inside the box showed no growth of microorganisms.

Based on MTT assay, plasma jet exhibited cancer-killing property as approximately 57 % of breast cancer cells were dead after APAPJ treatment for 60 seconds. As significant thermal effects and pH change were not observed in the DMEM cell culture media (Figure 2), we reckon that plasma species generated in APAPJ are solely responsible for the killing effect in cancer cells. The further biochemical and analytical test is essential to confirm the type of plasma species responsible for killing and mechanism behind the death of cancer cells.

### **Enhancement of cell adhesion and proliferation by plasma jet**

Significant cell adhesion, growth, and proliferation were observed and eventually confirmed by CV staining when breast cancer cell-line was grown after treating polystyrene Petri dishes with APAPJ (Figure 3B). To investigate the surface modifying property of APAPJ used to treat the polystyrene substrate in the experiment, the contact angle was measured after the treatment. There was a significant decrease in contact angle from 35.2°±2 to 30.3°±3 (Figure 3A) when treated with APAPJ for 60 to 240 seconds (Table 1).

The decrease in the contact angle verified the modification of polystyrene surface towards hydrophilic nature, which is meant to enhance the cell adhesion. Implantation of negatively charged species such as -COOH group has been found to enhance cell adhesion by increasing the hydrophilicity of the cell culture medium containing cells [10].

The effect of APAPJ treatment in the polystyrene petri dish to enhance cell adhesion and proliferation was further quantified by MTT assay which showed significant growth of breast cancer cell-lines after 48 hours of incubation (Figure 4). APAPJ treatment of polystyrene Petri dishes for 240 seconds enhanced adhesion, growth, and proliferation of viable cells by approximately 50%.

### **Conclusion**

We conclude that the plasma jet is solely responsible for killing breast cancer cells because significant changes in pH and temperature were not observed in the culture medium. The electron temperature of 0.372 eV indicates that the plasma generated in our laboratory is non-thermal or cold and can be used for treating cells and tissue. As future aspects, we are intending to treat different cancer cell lines along with normal cell lines by APAPJ to study plasma selectivity in cancer therapy. We also intend to perform further biological assays to observe cell growth kinetics, morphological change and colony survival assay. In the future, various physical and chemical parameters such as gas flow rate, distance between jet nozzle and cell, electric arcs in the jet plume and reactive species generated in culture media will be optimized. Most importantly, further biochemical and molecular tests will be conducted to figure out whether the cancer cells treated by our APAPJ are dead due to traumatic injury i.e. necrosis or by programmed way i.e. apoptosis. APAPJ was used to treat cell culture substrates such as polystyrene petri dish to enhance cell adhesion and proliferation in animal cell culture laboratory. APAPJ can be designed in different geometrical configurations to treat an inexpensive petri dish. We observed that increased hydrophilicity enhanced cell adhesion and proliferation whereas ion implantation may have also expedient the cell growth is yet to be demonstrated. Also, these enhancement in cell adhesion and proliferation property induced by APAPJ treatment could be beneficial for tissue regeneration and organ culture. Thus, in conclusion, the atmospheric pressure plasma jet is emerging as a promising technology for biological sciences such as surface modification of biomaterials and cancer therapy.

### **Acknowledgment**

The authors would like to thank Mr. Sandeep

Adhikari Chhetri, a graduate student at Department of Biotechnology, Kathmandu University for his valuable guidance and suggestions in cell culture experiments.

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