Plasma Medicine, 2(1-3): 71–83 (2012)

Deep Penetration into Tissues of Reactive Oxygen Species Generated in Floating-Electrode Dielectric Barrier Discharge (FE-DBD): An *In Vitro* Agarose Gel Model Mimicking an Open Wound

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ABSTRACT: In this article we present an *in vitro* model based on agarose gel that can be used to simulate a dirty, oily, bloody, and morphologically complex surface of, for example, an open wound. We show this model's effectiveness in simulating the depth of penetration of reactive species generated in plasma (e.g., hydrogen peroxide) deep into the tissue of a rat and confirm the penetration depths using an agarose gel model. We envision that such a model could be used in the future to study plasma discharges (and other modalities) and minimize the use of live animals; plasma can be optimized on the agarose gel wound model and then finally verified using an actual wound.

KEY WORDS: plasma medicine, animal model, rat, dielectric barrier discharge

I. INTRODUCTION

Plasma medicine is now a rapidly advancing field with positive indications of plasma's ability to sterilize many types of surfaces,[1–4] including human and animal tissues[5]; treat wounds[6,7]; coagulate blood[5,8]; and even treat diseases.[7] This is mostly likely due to the plasma catalysis of the reduction/oxidation reactions happening in the biological system. The action of specific charged or neutral active species or radiation is frequently associated with the corresponding specific effect (e.g., the anti-inflammatory effect of nitric oxide and highly oxidative hydroxyl radicals and other reactive oxygen species). With the vast amount of recent research it is becoming clear that plasmas are indeed able to induce some perhaps positive clinical effects in patients. Thus, it is important to analyze not only plasma's effects on an organism but also the plasma itself. Clearly, plasma in contact with dirty, oily, bloody tissue is not the same as plasma generated between 2 perfectly controlled electrodes. For this reason we have developed a simple *in vitro*

1947-5764/12/$35.00

model in which an agarose gel is the second electrode used for plasma generation. In this article we compare measurements of species generated by plasma on an agarose gel to those on store-bought chicken meat and those on a live rat wound. Surprisingly, the findings reported here suggest that a properly prepared agarose gel may actually serve as a very good model of the penetration of plasma-generated species into the tissue. Indeed, if our findings are true and are confirmed by other research groups, we may begin to analyze plasma (performing spectroscopic, microwave, and other measurements) with a simple agarose gel model as the second electrode ; these measurements would be a sufficiently accurate representation of the real environment. We leave it to the reader to judge our findings and urge them to verify our results.

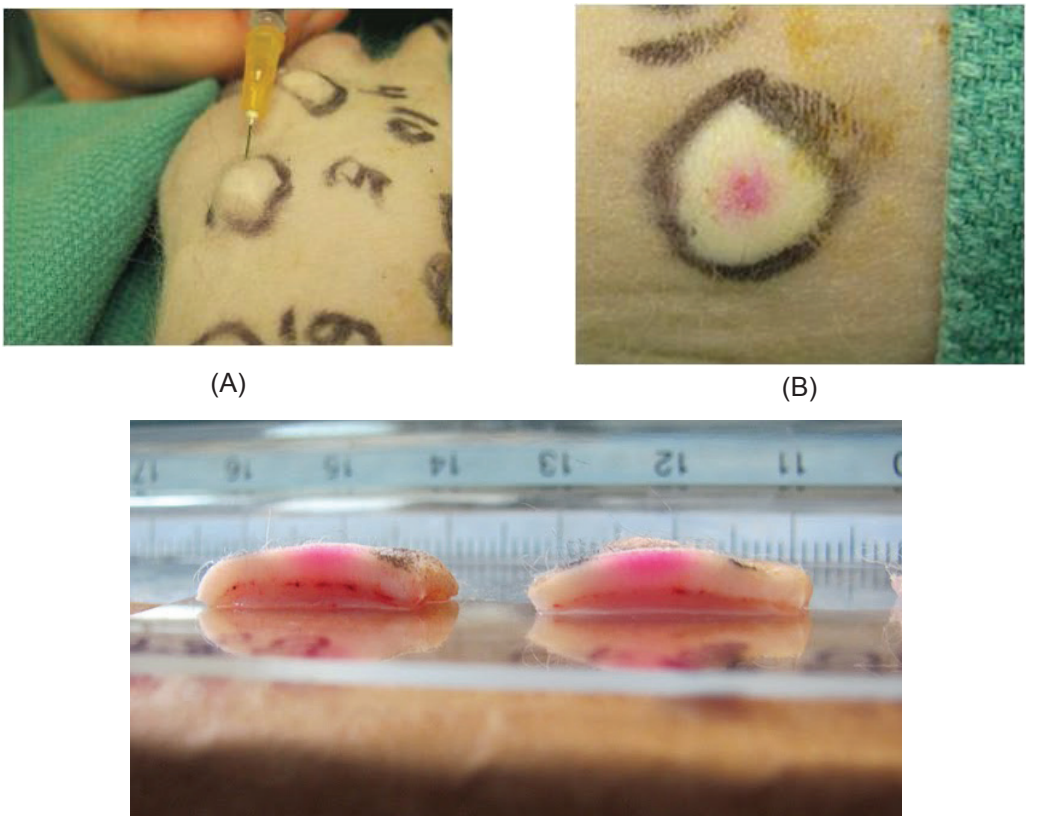
II. MATERIALS AND METHODS

In this study we used atmospheric pressure dielectric barrier discharge (DBD) plasma at room temperature in air. DBD plasma was generated using an experimental setup similar to one previously described elsewhere.[5–7,9,10] In short, the discharge was generated by applying an alternating polarity pulsed (1 kHz) voltage of ~20-kV magnitude (peak to peak) and a rise time of 5 V/ns between the insulated high-voltage electrode and the sample undergoing treatment. The powered electrode was made of a solid copper disc with a 1.5-cm diameter covered by a 1-mm-thick quartz dielectric with a 1.9-cm diameter. The discharge gap was kept at 1.5 mm. Duration of the current peak was 1.2 μs, and the corresponding plasma surface power density was 0.3 W/cm^2. In the case of *ex vivo* measurement in a rat tissue, a special pen-sized electrode was used: 1-mm-thick, polished, clear, fused quartz (Technical Glass Products, Painesville, OH) was used as an insulating dielectric barrier, and a handheld pen-like device with a quartz tip was used for treatment. In this case, the average power density for the active area of the high-voltage electrode was kept at a level of approximately 0.74 W for an electrode with a 6-mm diameter.

Agarose gels were prepared using standard procedures with pure agar powder (Fisher Scientific) in either distilled water or phosphate-buffered saline (PBS) (Fisher Scientific). To determine the best concentration of agarose gels that would closely represent tissue, we used agar at concentrations of 0.6%, 1.5%, and 3% weight. These values were chosen for the following reasons. Agarose gels at 0.6% concentration were reported to closely resemble *in vivo* brain tissue with respect to several physical characteristics.[11] Four percent agar phantoms are widely used as tissue models for radiology studies.[12] The 1.5% concentration of agar was chosen as a median point that is often used as a microbiological substrate.

Hydrogen peroxide (H_2O_2) penetration into agarose gels (0.6%, 1.5%, and 3% wt) and tissues as well as the and pH of the gels were measured using Amplex UltraRed reagent (excitation wavelength, 530 nm; emission wavelength, 590 nm; Invitrogen) and fluorescein (excitation wavelength, 490 nm; emission wavelength 514 nm; Sigma Aldrich) fluorescent dyes, respectively. In the case of H_2O_2, 75 μL PBS containing 100 μM Amplex UltraRed with 200 U/μL horseradish peroxidase (MP Biomedicals) were placed between 1-mm-thick 4×4-cm agar slices and incubated for about 15 minutes before the treatment to ensure the presence of the dye in the agar volume; for the pH measurement, the agarose

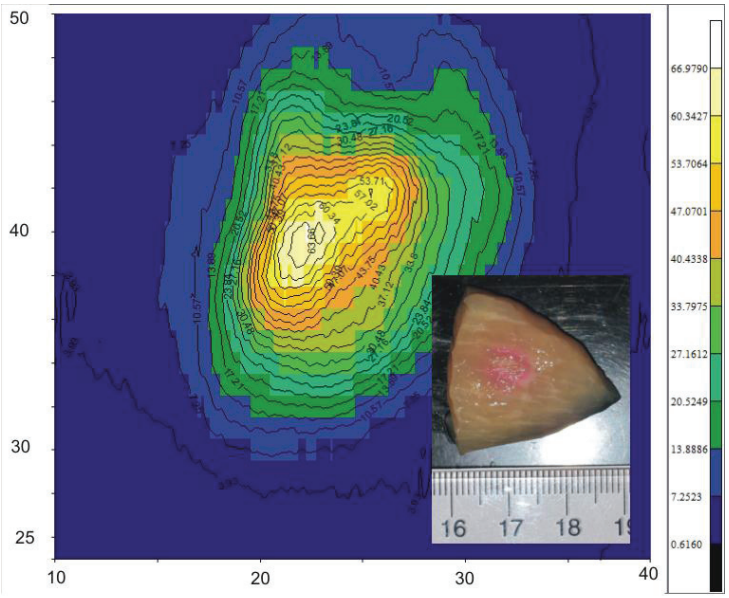
gels were prepared by adding fluorescein dye before they solidified. To measure the H_2O_2 in and the pH of tissue, the dyes were injected using a syringe into a 1-cm-thick 4×4-cm sample of skinless chicken breast tissue at various points to the depth of up to 1 cm. *Ex vivo* measurements were done in a rat tissue: a hairless Sprague-Dawley male rat was killed right before the procedure; 200 μL of dye solution (Amplex UltraRed) was injected subcutaneously using a sterile syringe, and the animal's skin was treated with floating electrode DBD plasma at various time points after a 5-minute incubation period (Fig. 1). Right after the treatment, samples of skin tissue were extracted and analyzed as follows. Treated samples were cut in a vertical direction into slices with a thickness of 1 mm; fluorescence was measured using an LS55 fluorescent spectrometer (Perkin Elmer) equipped with an XY reader accessory (Figs. 2 and 3). To obtain calibration curves for H_2O_2 in the plasma-treated samples, a properly diluted standard stabilized 3% H_2O_2 water solution (Fisher Scientific) was used at various concentrations.



(A) (B)

(C)

FIG. 1: Subcutaneous injection of the fluorescent dye into the euthanized rat **(A)**, the rat skin after the plasma treatment **(B)**, and the cross-section of the skin sample just before measurement **(C)**.



(A)

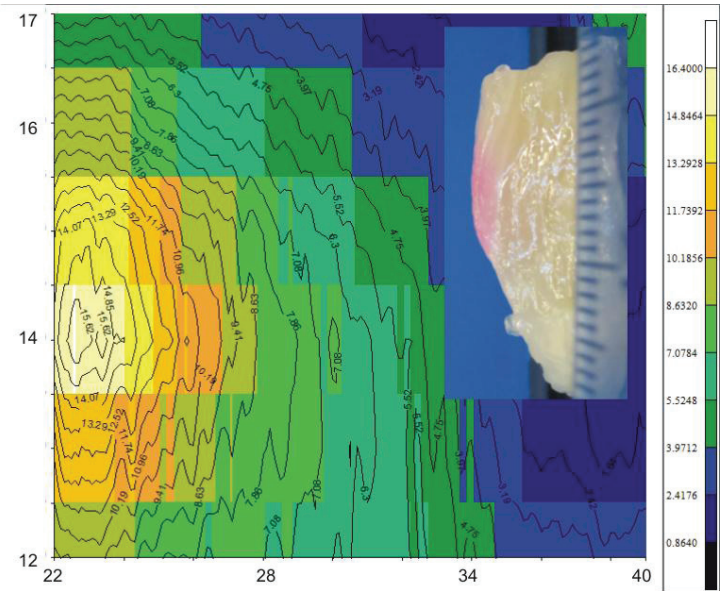


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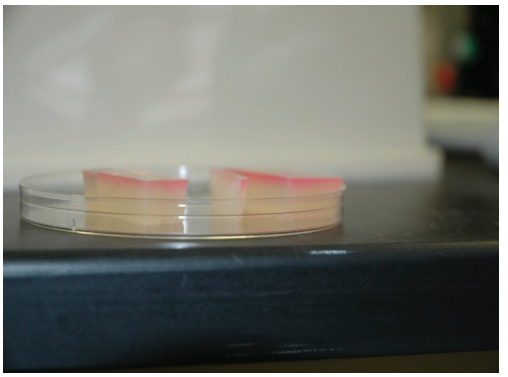
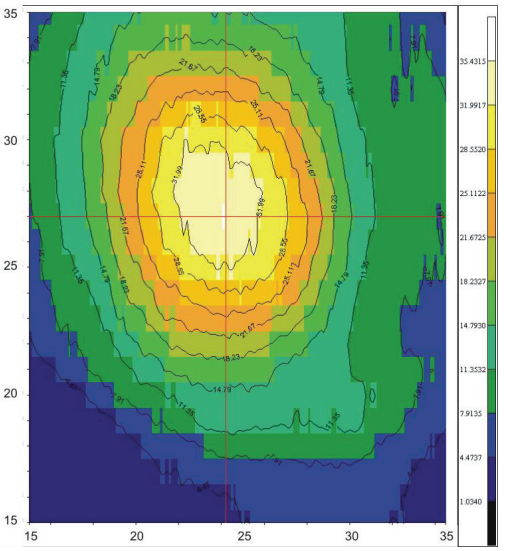
FIG. 2: Chicken breast after plasma treatment with hydrogen peroxide fluorescent dye: photograph and fluorescent images from the top (left) and side (right) of the sample (color intensity in arbitrary units, dimensions in millimeters).



(A)



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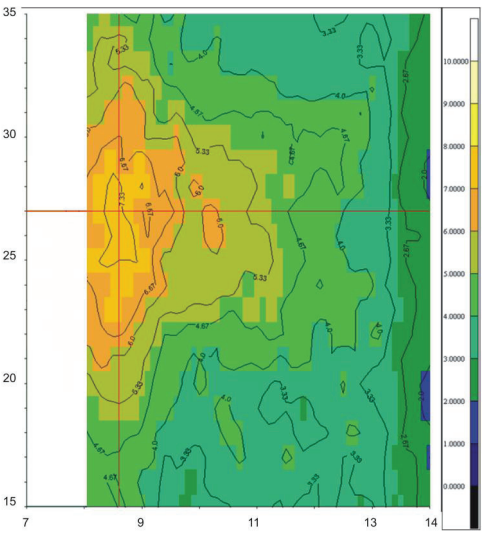


(C)

FIG. 3: Agarose gel after plasma treatment with hydrogen peroxide fluorescent dye **(A)** and photograph and fluorescent images from the top **(B)** and side **(C)** of the sample (arbitrary units, dimensions in millimeters).

III. RESULTS

The results of H_2O_2 measurements in dead tissue are shown on Fig. 4: the depth of penetration as well as the concentrations of H_2O_2 increased with longer treatment time. In general, several millimoles of H_2O_2 are produced in tissue after plasma treatment, while it diffuses at depths of 1.5–3.5 mm. Roughly the same tendency is observed in the case of tissue acidity change, shown in Fig. 5 (florescence intensity of fluorescein decreases

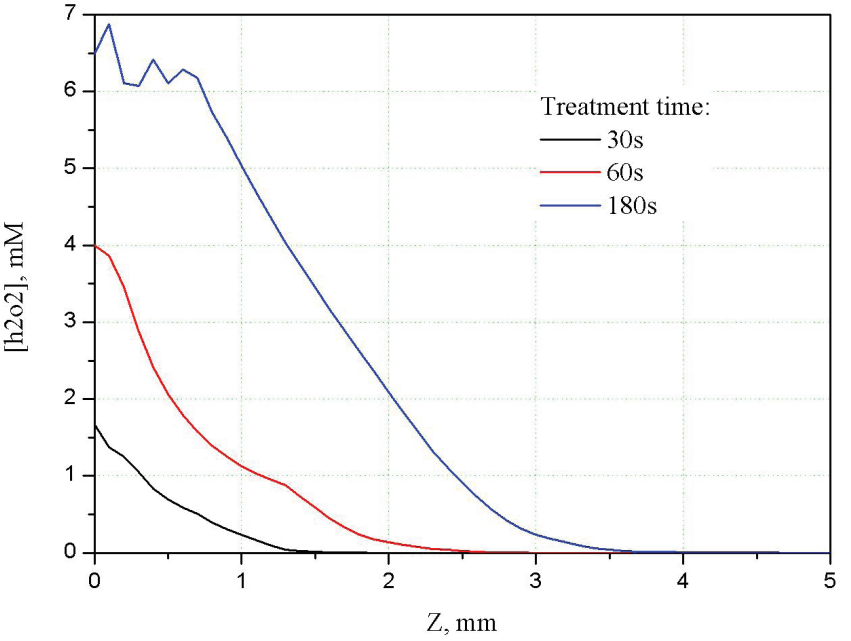


FIG. 4: The profiles of hydrogen peroxide (H_2O_2) in tissue after the plasma treatment.

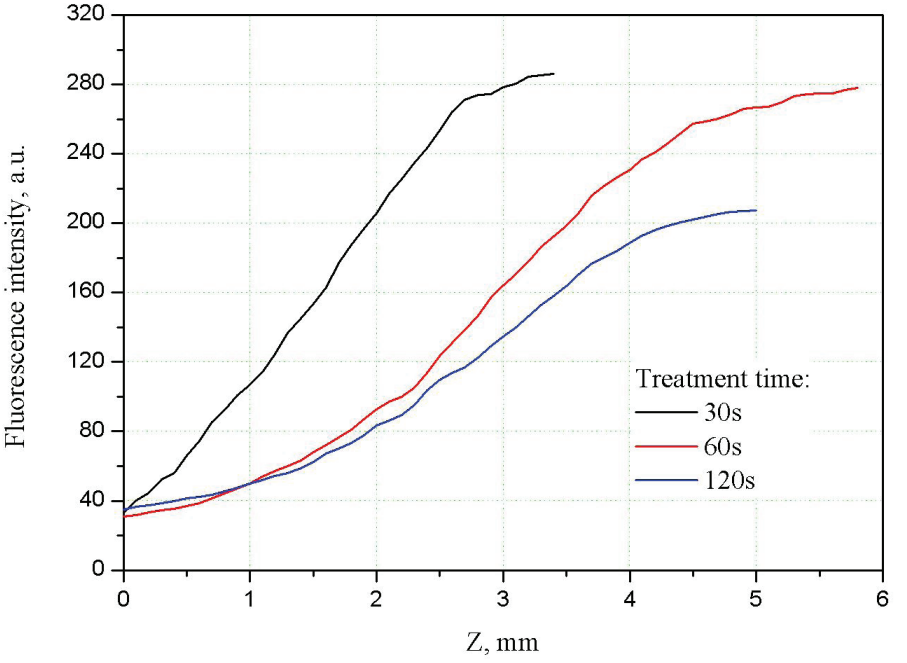


FIG. 5: The profiles of pH in dead tissue after the plasma treatment.

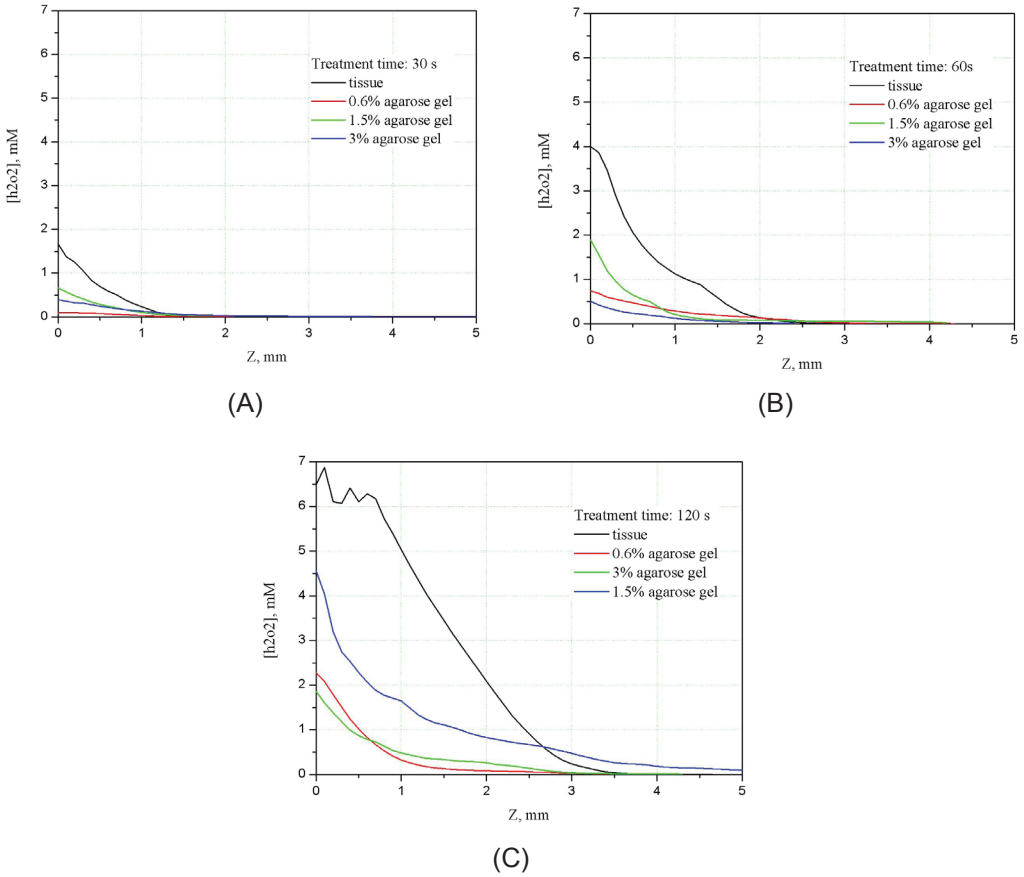


FIG. 6: The profiles of hydrogen peroxide (H_2O_2) in nonbuffered agarose gels and dead tissues after 30-second **(A)**, 60-second **(B)**, and 120-second plasma treatment **(C)**.

with lower pH, and the data are presented as arbitrary units); however, the effect of pH lowering penetrates deeper—up to 4.5–5 mm.

To develop a simple realistic *in vitro* model of tissue that would have simple physicochemical characteristics, primarily from the point of view of depth of penetration of reactive species, 3 concentrations of agarose media were used. The measurement results for H_2O_2 produced by plasma treatment in agar gels together with the results for dead tissues for the same treatment doses are shown in Fig. 6. H_2O_2 concentration on the agar gel surface varied with different agar densities: 0.5 mM for 0.6%, 0.7 mM for 3%, and 1.9 mM for 1.5% gels after 1 minute of plasma treatment. The results of H_2O_2 penetration measurements in rat skin tissues are shown in Fig. 7; compared to the dead chicken breast tissue, depth of penetration seems to be very similar: up to 4 mm after a 2-minute treatment (although the concentration is almost an order of magnitude greater because the power of the discharge is 8 times higher). Surprisingly, depth of H_2O_2 penetration

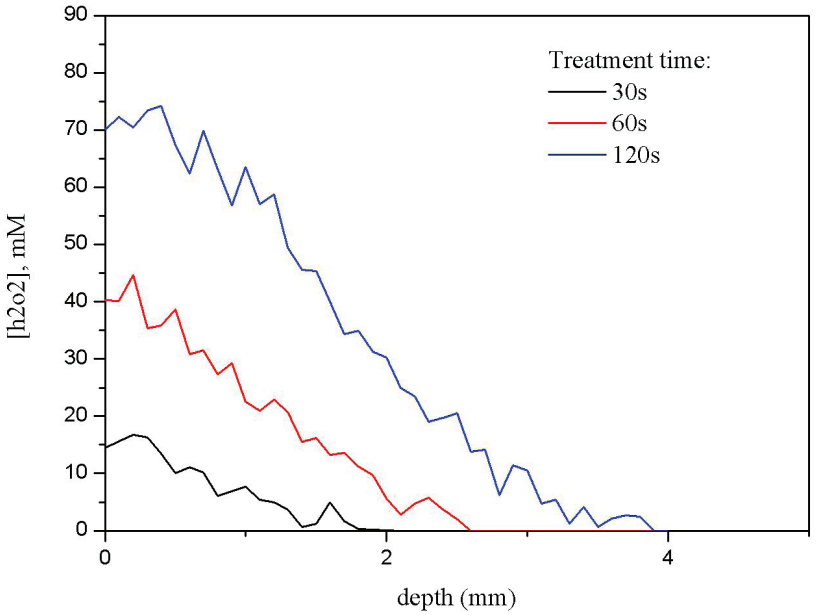


FIG. 7: The profiles of hydrogen peroxide (H2O2) in tissue from euthanized rats after the plasma treatment.

for all types of agarose media was about the same; Fig. 8 shows the depths at which the same level of 0.05 mM of H_2O_2 was detected in agar gels and tissue for different plasma exposure times.

In contrast to H_2O_2, the dynamics of acidity change inside of agar gels fluctuate significantly with each agar media composition (Fig. 9). Tissue seemed to have better buffer properties compared to nonbuffered agarose gels. However, similarities in terms of pH changes were expected with the addition of PBS into agar displays.

IV. DISCUSSION

In this article we address a problem of direct atmospheric pressure plasma interaction with tissues, in particular the delivery of neutral active components produced by plasma inside of tissues and the creation of a physical model of such interaction. Plasma is a complex chemically active medium, and a number of biomedical studies suggest that it can be successfully applied to various living tissues without damage,[13–15] even providing a number of positive effects, such as blood coagulation,[5,7,8,16] wound treatment,[7,16–18] and even cancer treatment.[10,19,20] Notably, for instance, in the case of experimental subcutaneous cancer tumor treatment in a mouse model,[20] the effect of plasma treatment was observed not only on the surface of treated tissue (skin) but also (and chiefly) in volume under the skin. In many cases, researchers report a significant change of pH of solutions and tissues after plasma treatment (see, e.g., Refs. 20 and 21). On the other hand,

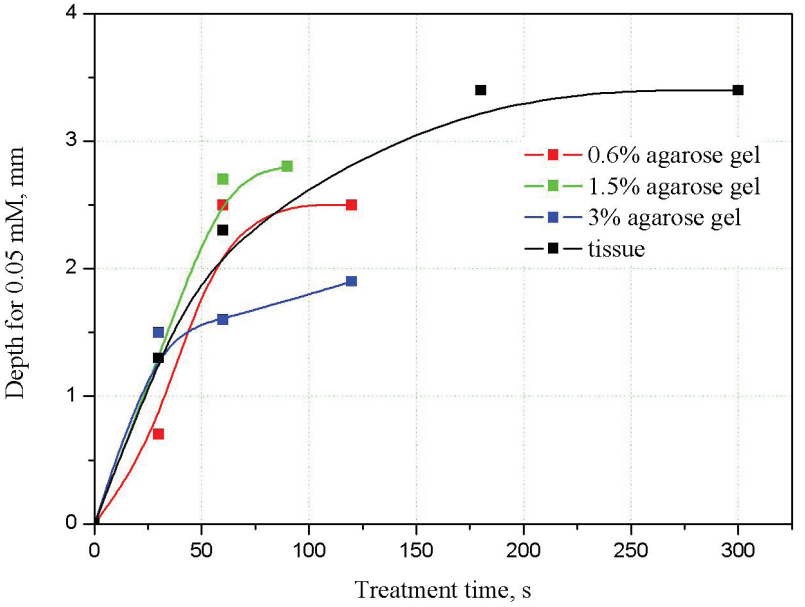
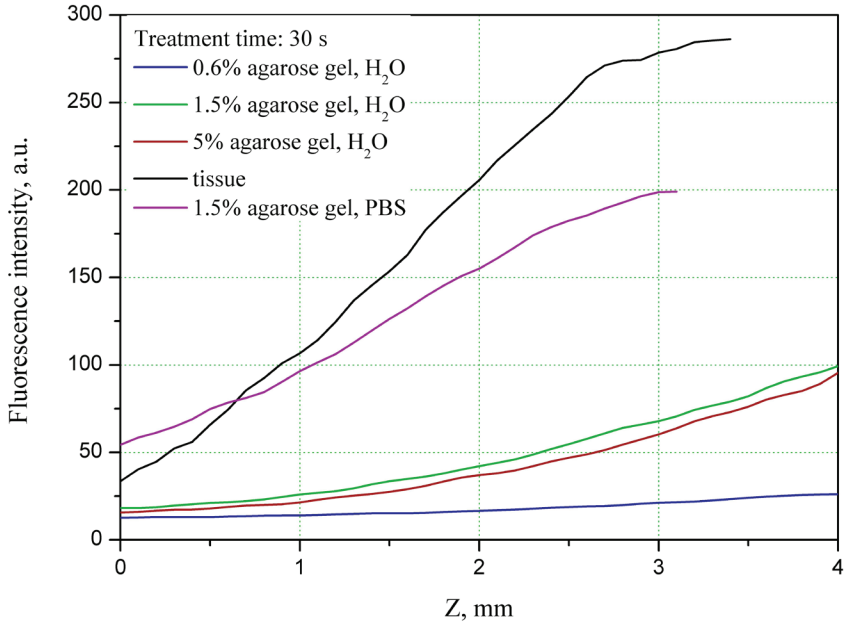


FIG. 8: Depth of hydrogen peroxide penetration at concentration of 0.05 mM in both agarose gels and dead tissue.

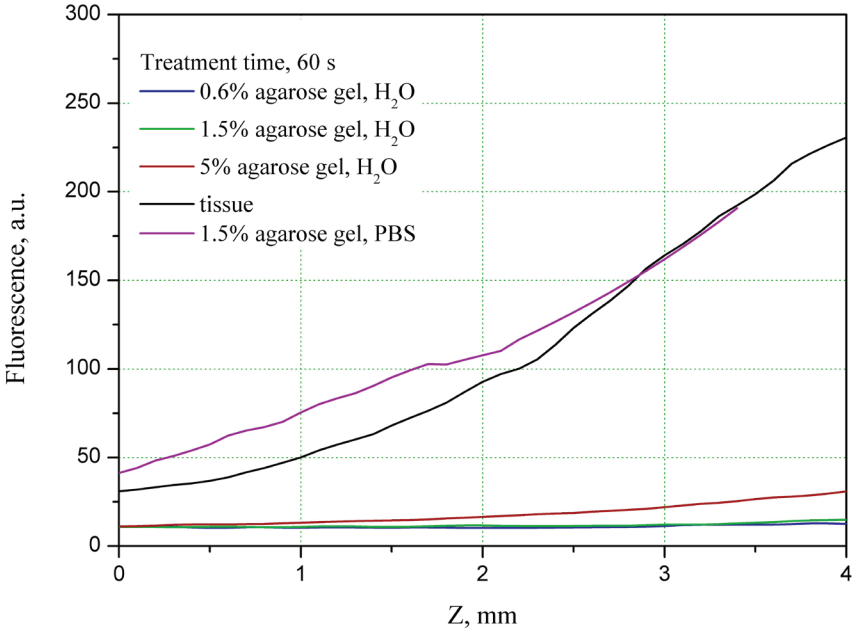
H_2O_2, which is almost always produced by plasma, is often considered to be important in bacteria inactivation[6,21–24] and wound healing processes.[25] Therefore, in this study we focused on measurements of these 2 parameters—pH and H_2O_2 concentration—as a function of treatment time and depth of penetration into a treated object.

The evaluation of measuring techniques of the effects of plasma treatment on tissues is complicated in direct experiments on actual biological objects because of a wide variation of morphological and biochemical parameters that are beyond the control of the researcher. Therefore, stable and reproducible test objects that mimic tissues' physicochemical characteristics are needed. In this study we used a custom-made agarose gel similar to those used in microbiological studies but modified to achieve certain similarities to dead tissue. Agarose phantoms are widely used as models of various tissues (see, e.g., Refs. 11, 12, 26, and 27).

The results we present in this article show that, in the case of real tissue, active species produced by plasma on the surface may travel in tissue volume to a depth of several millimeters. This depth of penetration is obviously determined by diffusion and reaction rates, which would highly depend on the type of tissue and its biochemical characteristics. A measurement of these parameters is an extremely complicated task; therefore, creation of a simple model that would closely represent certain tissue is possible experimentally. We used agarose gels of various concentrations to mimic the physical properties of tissue. For the case of H_2O_2, as shown in Figs. 6 and 8, the depth of diffusion is about the same for all types of agar, but the concentration of H_2O_2 varies,



(A)



(B)

FIG. 9: The profiles of pH in agarose gels and dead tissues after 30-second **(A)** and 60-second **(B)** plasma treatment.

being the closest for the 1.5% wt agar gel and about 3 times lower for both 0.6% and 3% wt agar gels. This behavior may possibly be explained by both diffusion properties and reaction rates of H_2O_2 in agar.

The acidity of tissue in our experiments was observed to consistently increase (lowering of pH) as a result of exposure to the discharge, in contrast to the experimental *in vivo* data reported by Melly et al.,[24] who observed a significant decrease in pH on the skin's surface with a slight increase in the subcutaneous tissue. Since the fluorescence of fluorescein highly depends not only on pH of a solution but also on other parameters,[28–32] we did not attempt to obtain calibration curves; therefore the data are presented in arbitrary units. We show that tissue compared to agarose gels prepared in distilled water acts as a significantly better buffer and that the pH changes at various depths inside such gels are much greater. In fact, we observed a significant drop in the whole volume of a phantom—up to a 1-cm-thick agar. This problem, as shown in Fig. 9, may be addressed simply by adding a buffer into the agarose media: 1.5% wt agar was prepared in 1X PBS.

In summary, we show that plasma effects may be transferred several millimeters deep inside a tissue, as measured in *ex vivo* chicken tissue and rat skin models. We detected penetration behavior of 2 simple active components, namely H_2O_2 and pH, but other species may be detected and measured using other fluorescent dyes or techniques. In addition, we have shown that a simple agar gel model may express physicochemical properties similar to those of real tissue, resulting in comparable penetration effects of active species.

V. CONCLUSION

In this study we used atmospheric pressure DBD treatment of living rat tissue and developed a corresponding agarose gel model that mimics the tissue. Our findings suggest that a carefully prepared agarose gel may serve as a very good model of penetration of plasma-generated active species (such as reactive oxygen and reactive nitrogen species) into the tissue. We show that this simple model can be accurately used now for further plasma studies (spectroscopic measurements, microwave diagnostics, etc.) without the need to use a live animal for each experiment. This may potentially significantly reduce the regulatory hurdles in performing plasma medical experiments, reduce the number of animals needed to complete a study, and ultimately generate a larger bank of experimental data. The data on penetration on H_2O_2 into the animal tissue reported here can be reproduced with a custom-made agarose gel. These findings need to be validated for other plasma-generated active species as well, and this work is currently underway.

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