

# Mechanism of Blood Coagulation by Nonthermal Atmospheric Pressure Dielectric Barrier Discharge Plasma

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**Abstract**—Mechanisms of blood coagulation by direct contact of nonthermal atmospheric pressure dielectric barrier discharge (DBD) plasma are investigated. This paper shows that no significant changes occur in the pH or  $\text{Ca}^{2+}$  concentration of blood during discharge treatment. Thermal effects and electric field effects are also shown to be negligible. Investigating the hypothesis that the discharge treatment acts directly on blood protein factors involved in coagulation, we demonstrate aggregation of fibrinogen, an important coagulation factor, with no effect on albumin. We conclude that direct DBD treatment triggers selective natural mechanisms of blood coagulation.

**Index Terms**—Blood coagulation, dielectric barrier discharge (DBD), nonthermal plasma, plasma medicine.

## I. INTRODUCTION

OVER the past few years, nonthermal atmospheric pressure plasma has emerged as a novel promising tool in medicine. As compared to the effects of the more conventional thermal plasma [1], nonthermal plasma is selective in its treatment since it does not burn tissue. This enables many new medical applications, including sterilization of living tissue without damage [2], blood coagulation [2], induction of apoptosis in malignant tissues, [3], [4] and modulation of cell attachment [5]–[7].

Two different approaches to the use of nonthermal plasma effects in medicine have been pursued. In one approach, plasma

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is created remotely, and its afterglow is delivered by a jet to the desired location. In this indirect approach, relatively long-living active plasma species do most of the desired work, whereas most of the charged particles do not survive outside the plasma generation region. Alternatively, nonthermal atmospheric pressure plasma has been generated in direct contact with living tissue. Here, it has been shown that treatment produces various effects much faster due to direct contact with charged species [8].

Although blood coagulation by direct nonthermal plasma treatment has been reported before [2], the biochemical pathways (mechanisms) through which such coagulation occurs remain largely unclear. In this paper, several possible mechanisms are investigated. We demonstrate that direct plasma triggers natural, rather than thermally induced, coagulation processes. We also demonstrate that release of  $\text{Ca}^{2+}$  and changes of blood pH level are insignificant. Instead, the evidence points to selective action of direct nonthermal plasma treatment on blood proteins involved in the natural coagulation processes.

The principle of operation of the discharge used in this paper is similar to the dielectric barrier discharges (DBDs) introduced by Siemens [9] in the middle of the 19th century. DBD occurs at atmospheric pressure in air or other gases when sufficiently high voltage of sinusoidal waveform or pulses of short duration are applied between two electrodes, when at least one of them is insulated [10]. The presence of an insulator between the electrodes prevents the buildup of high current. As a result, the discharge creates e-plasma (we use this term to avoid confusion with blood plasma) without substantial heating of the gas. This approach allows for treatment of biological samples without thermal damage while biological processes are initiated and/or catalyzed with the help of electrical charges [8].

## II. MATERIALS, METHODS, AND EXPERIMENTAL SETUP

In this paper, we investigate mechanisms of blood coagulation by nonthermal atmospheric pressure plasma using an experimental setup similar to one previously described by the authors [2] and schematically illustrated in Fig. 1. E-plasma is generated by applying alternating polarity pulsed voltage of  $\sim 35$ -kV magnitude (peak to peak) at 1-kHz frequency between the insulated high-voltage electrode and the sample undergoing treatment. One-millimeter-thick polished clear fused quartz is used as an insulating dielectric barrier. The discharge gap between the bottom of the 1-mm-thick quartz glass covering the

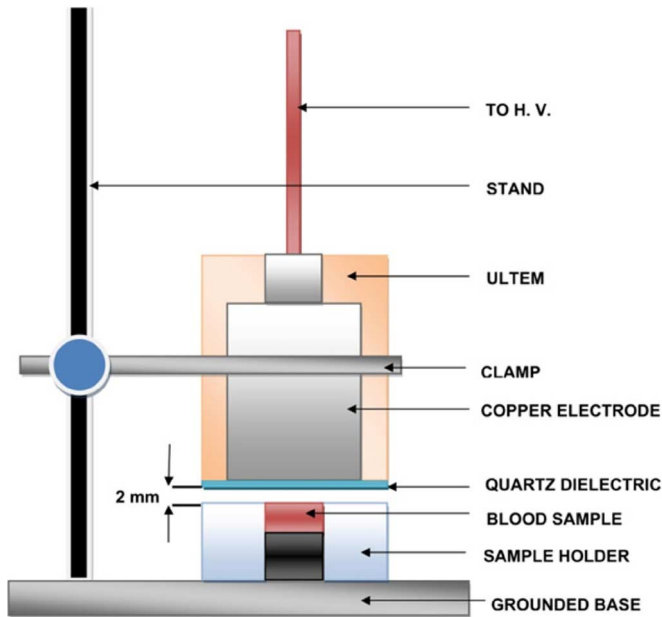


Fig. 1. Schematic of the experimental setup showing the high-voltage electrode and the sample holder.

copper electrode and the top surface of the sample being treated was set to 2 mm. The diameter of the copper electrode that was employed was 2.5 cm. The power density of the discharge has been measured to be around  $1.5 \text{ W/cm}^2$  using both electrical characterization and special calorimetric setup [11].

All the treatments are at room temperature and atmospheric pressure and were carried out according to the same protocol. The control samples were placed in the same sample holder as the treated sample. The control sample was exposed to air for the same time as the treated samples were exposed to plasma. For plasma treatment of  $500 \mu\text{L}$  of anticoagulated whole blood, a special sample holder was constructed. A 25.4-mm-tall polycarbonate plate was used as base, and stainless steel rods were inserted into a 25.4-mm through hole. Stainless steel rods (21.46 mm tall) were used. For this paper, deidentified whole-blood samples with three different types of anticoagulants were obtained from the Chemistry Laboratory, Drexel University College of Medicine. Five hundred microliters of the three different types of anticoagulated whole blood, viz., heparinized whole blood, citrated whole blood, and ethylenediaminetetraacetic acid whole blood, were used. Each blood sample was treated for 5, 15, 30, and 60 s to study the effect of e-plasma treatment. Changes in pH and  $\text{Ca}^{2+}$  concentration were determined immediately after treatment. The pH of the sample was measured using a pH meter (Lazar Research Laboratories, 6230n pH/mV/Temp meter) and a pH microelectrode (Lazar Research Laboratories, PHR146XS).  $\text{Ca}^{2+}$  concentration was measured after the treatment using the said meter with a micro-ion-selective electrode (Lazar Research Laboratories, LIS-146CACM) and a microreference electrode (Lazar Research Laboratories, LIS 146DJM). The obtained millivolt values were converted to molar values using software (Arrow Laboratory Systems).

To study the effect of the nonthermal plasma exposure on albumin and fibrinogen, which are constituent proteins of blood

plasma, purified lyophilized human serum albumin (Sigma-Aldrich, St. Louis, MO) was dissolved in trishydroxymethylaminomethane (TRIS)-buffered saline to obtain an albumin solution (physiological concentration of 2 g/L) at a physiological pH of 7.4. To prepare a fibrinogen solution, purified lyophilized fibrinogen from human plasma (Sigma-Aldrich, St. Louis, MO) was layered on top of warm ( $37^\circ\text{C}$ ) 20 mM TRIS-buffered saline (Sigma-Aldrich) and slowly agitated for 2 h to obtain a fibrinogen solution (physiological concentration of 30 g/l) at a physiological pH of 7.4.

To determine the effects of electric field on blood coagulation by e-plasma treatment, 0.5 ml of anticoagulated whole blood was treated by covering the surface of blood by 0.17- to 0.25-mm-thick glass coverslips (Fisher Scientific, Inc.) and then with a 0.13-mm-thick plastic transparency film. The coverslips and plastic transparency film serve as a screen to all the active elements of e-plasma, except electric field and UV-C. This treatment was compared to the treatment of blood without covering it.

To determine the effects of average heating due to e-plasma treatment, 0.5 ml of blood was treated by covering the surface of blood with a 25-micrometer to 40- $\mu\text{m}$ -thick aluminum foil. The aluminum foil serves as a screen to the active elements of the e-plasma while transferring the heat from the e-plasma discharge to the sample. This treatment was compared to the treatment of blood without the aluminum foil cover.

Morphological evaluation of the clot layer, which was formed on the surface of blood post e-plasma treatment, was conducted at the Drexel Material Characterization Facility. The blood sample was treated for 30 s by e-plasma using the setup illustrated in Fig. 1. After the treatment, the clot layer was gently transferred onto a silicon wafer and immediately fixed overnight in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 [12]–[14]. The clot layer was then washed four times in distilled water and dehydrated in a graded series of increasing ethanol concentration (30%–100%) over 2 h. The specimens were mounted, sputter coated with platinum palladium in a scanning electron microscope (SEM) coating unit for 15 s, and examined in a Philips XL30 field-emission environmental SEM.

Dynamic light scattering (DLS) [15], [16] was used to detect aggregation of protein solutions (fibrinogen and albumin) due to e-plasma treatment. DLS-measured particle-size distributions in fibrinogen and albumin solutions before and after e-plasma treatment were obtained using Zetasizer NanoZS (Malvern Instruments). The Zetasizer uses a 633-nm He–Ne laser to measure the intensity of the light scattered due to the proteins and protein aggregates present in the solution.

### III. RESULTS AND DISCUSSION

Evaluated visually, a drop of blood, with a volume of  $500 \mu\text{L}$ , drawn from a healthy donor and left on a stainless steel surface coagulates on its own in about 15 min, whereas a similar drop treated for 15 s by e-plasma coagulates in under 1 min, as shown in Fig. 2(a). Similarly, 0.5 ml of citrated whole blood left in a well does not coagulate on its own even when left in the open air for well over 15 min, whereas the same sample

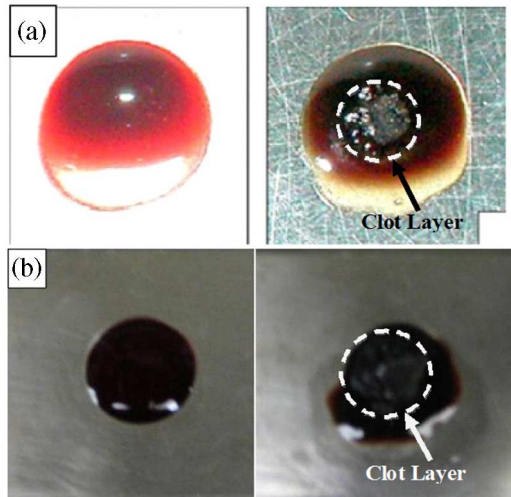


Fig. 2. Coagulation of the e-plasma-treated nonanticoagulated whole blood and citrated whole blood. (a) Nonanticoagulated donor blood treated with e-plasma for 15 s exhibits an immediate clot layer formation. (b) Citrated whole blood treated with e-plasma for 15 s exhibits an immediate clot layer formation.

treated with DBD plasma for 15 s exhibits an immediate clot layer formation on the surface exposed to plasma discharge, as shown in Fig. 2(b).

Although the discharge employed here is inherently non-thermal, transfer of some thermal energy to the sample could occur. To test the possibility that this could trigger coagulation of normal and anticoagulated blood, the following experiment was performed: The sample was covered by a thin sheet of aluminum foil (25  $\mu\text{m}$  thin). The aluminum foil cover provided a good thermal contact to the blood samples while effectively insulating it from the flux of UV, various active species, and charges. Both covered and uncovered control samples were treated for time periods that extended to over 30 s. The uncovered blood sample [Fig. 3(a) and (b)] showed a clot layer formation upon e-plasma treatment, whereas the sample covered with aluminum foil [Fig. 3(c) and (d)] did not exhibit a clot formation. This suggests that a natural process of clotting may be triggered by e-plasma, not just the transfer of thermal energy to the sample as would occur in cauterization of a blood vessel.

It is possible that the applied electric field may electrolyze the sample being treated and, thus, coagulate blood. To test this possibility, the following experiment was performed: First, anticoagulated whole blood was covered by glass coverslips (0.25 mm thick). The coverslips were in good contact with the blood sample and served as a screen to all the active elements and charges of e-plasma, except for the applied electric field and UV-C. Both covered and uncovered (control) samples were treated for time periods that extended to over 30 s. The uncovered blood sample showed a clot layer formation upon e-plasma treatment, whereas the sample covered with the coverslip did not exhibit any clot formation. A similar experiment was repeated by covering the sample with a plastic transparency film (4 mil in thickness). Again, the covered sample did not exhibit any clot layer formation. This suggests that the applied electric field alone is not responsible for blood coagulation.

To gain further insight into the structure of the clot layer of the anticoagulated blood samples, morphological examination

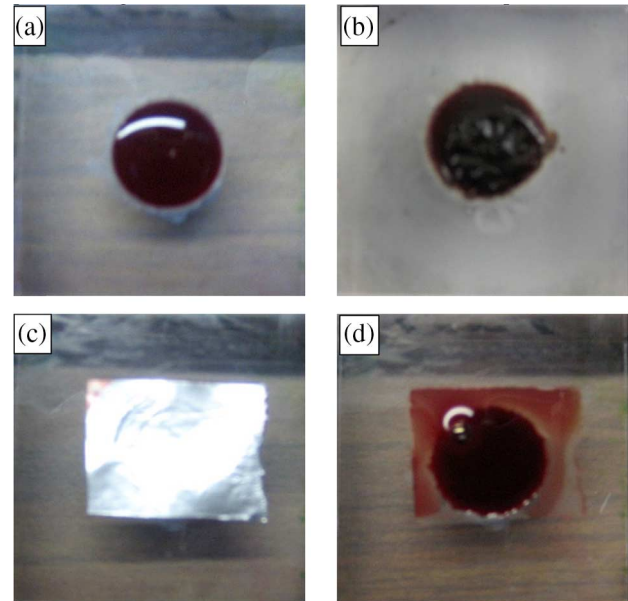
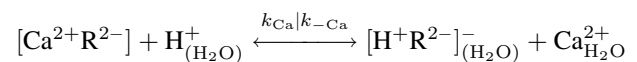


Fig. 3. (a) and (b) Uncovered citrated whole blood exhibits a clot layer formation after e-plasma treatment for 30 s. (c) and (d) Citrated whole blood covered with aluminum foil exhibits no clot formation after e-plasma treatment for 30 s.

of the clot layer was performed by SEM. Fig. 4(a) shows a single platelet [12], [17] over a red blood cell in an untreated (control) citrated whole blood. Fig. 4(b) shows some activated, but many nonactivated, platelets and red blood cells in the untreated anticoagulated whole blood. No platelet aggregation or fibrin strands are observed in these samples. On the other hand, extensive platelet activation (pseudopodia formation) and platelet aggregation were observed in the e-plasma-treated (30 s) whole blood, as evident in Fig. 4(c). Fig. 4(d) shows platelet activation, platelet aggregation, and fibrin formation (white arrows) in the e-plasma-treated anticoagulated whole blood.

Natural blood coagulation is a complex process that has been studied extensively [18], and various nonthermal plasma products can affect this process at many of the steps illustrated in Fig. 5 [19]. It was hypothesized previously that direct exposure to e-plasma initiates coagulation of blood through the increase in concentration of  $\text{Ca}^{2+}$  [2], an important factor in the coagulation cascade, as evident in Fig. 5 [20], [21]. Calcium circulates in blood in several forms: 45%–50% is free ionized, 40%–45% is bound to proteins (mostly albumin), and the rest is bound to anions such as bicarbonate, citrate, phosphate, and lactate [22]. Bound calcium is in equilibrium with free calcium. pH has a significant effect on calcium ion binding to protein, with each unit decrease or increase in pH causing ionized calcium to change inversely by about 0.36 mmol/L [23]. It was proposed that e-plasma is effective in the increase of  $\text{Ca}^{2+}$  concentration through a reduction/oxidation (redox) mechanism



provided by hydrogen ions generated through a sequence of ion molecular processes [24]. (Here, R represents the calcium-binding protein complexes such S100A7 [25] and albumin [26]–[28], for example.)

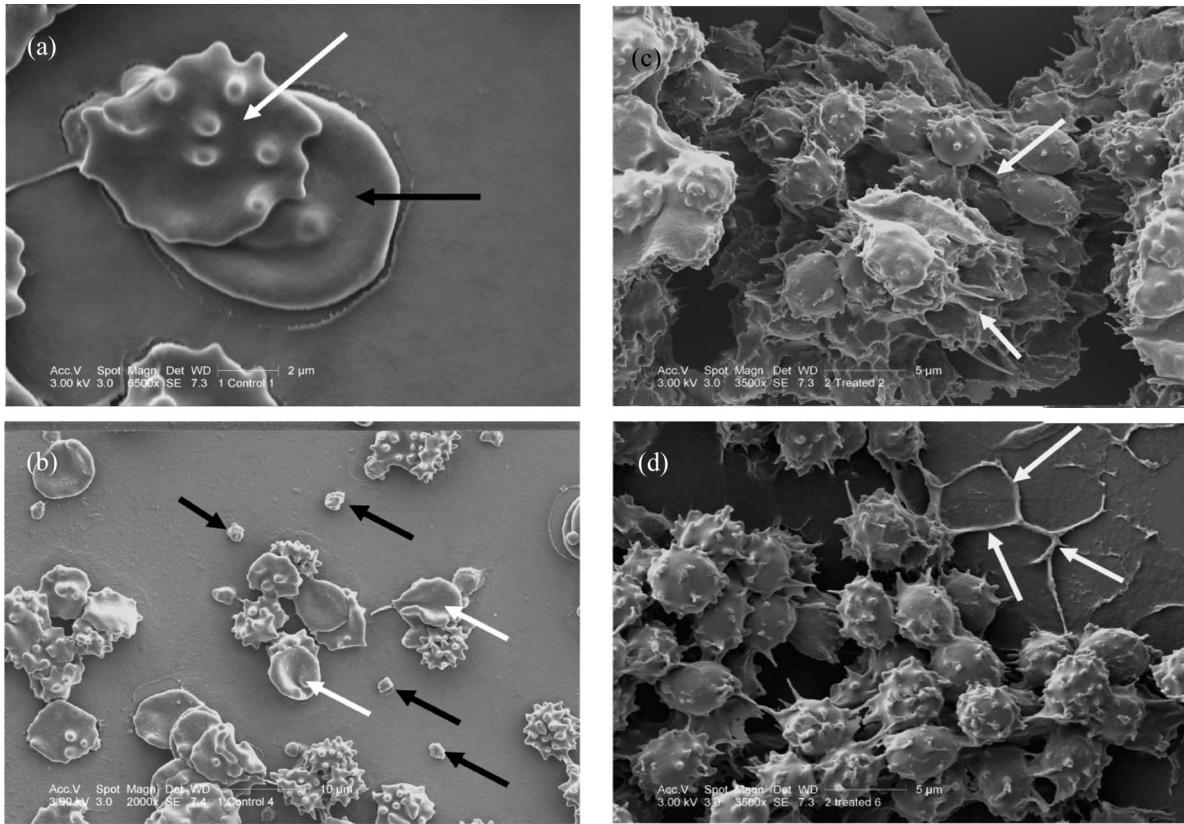


Fig. 4. (a) Citrated whole blood (control) showing a single activated platelet (white arrow) on a red blood cell (black arrow). (b) Citrated whole blood (control) showing many nonactivated platelets (black arrows) and intact red blood cells (white arrows). (c) Citrated whole blood (treated) showing extensive platelet activation (pseudopodia formation) and platelet aggregation (white arrows). (d) Citrated whole blood (treated) showing platelet aggregation and fibrin formation (white arrows).

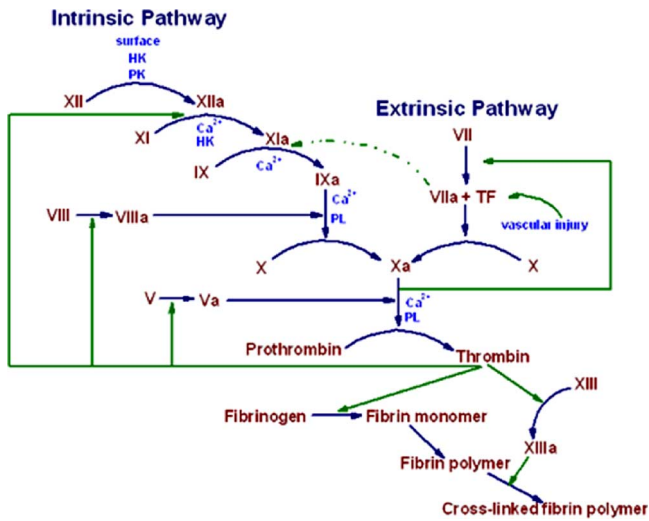


Fig. 5. Simplified coagulation cascade shows the dependence of most reactions on calcium ion  $Ca^{2+}$ .

We experimentally tested the validity of this hypothesis by measuring the  $Ca^{2+}$  concentration in the e-plasma-treated anticoagulated whole blood using a calcium-selective micro-electrode, as described in Section II. Calcium concentration was measured immediately after e-plasma treatment for 5, 15, 30, 60, and 120 s. Fig. 6 demonstrates that calcium concentration remains almost constant for up to 30 s of treatment and then increases very slightly for the prolonged

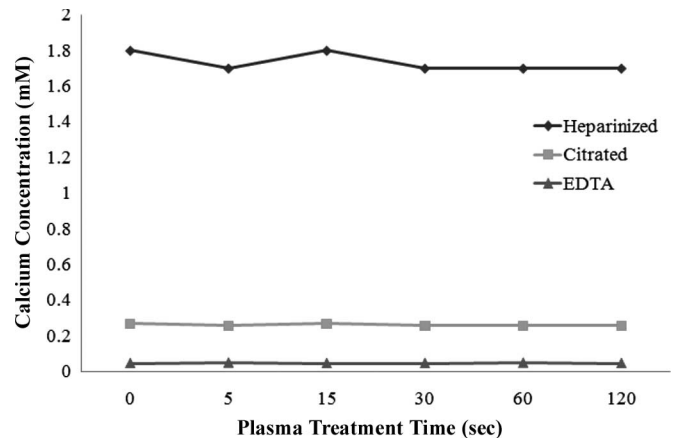


Fig. 6. Calcium concentration in different types of anticoagulated whole blood treated with e-plasma. There is no significant change in calcium ion concentration during the time e-plasma-treated blood coagulates. (Note: Average error is less than 0.01 mM.)

treatment times of 60 and 120 s. Thus, although e-plasma is capable of coagulating anticoagulated blood within 15 s, no significant change occurs in calcium ion concentration during this time in the discharge treated blood. Therefore, we conclude that the release of  $Ca^{2+}$  may not be the mechanism for the e-plasma-triggered coagulation.

Changes in blood pH could also trigger blood coagulation through the increase in calcium ion concentration due to the

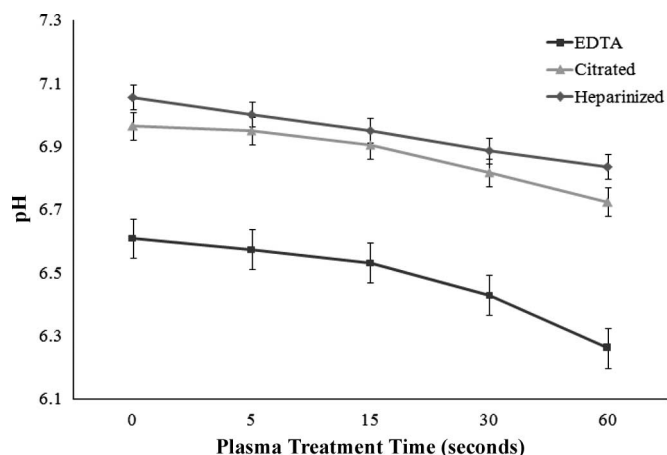


Fig. 7. pH of whole blood after e-plasma treatment for different durations. pH does not change significantly in the duration in which plasma-treated blood coagulates.

redox mechanism [2]. *In vivo*, the pH of blood is maintained in a very narrow range of 7.35–7.45 by various physiological processes [19].

E-plasma has been confirmed to generate a significant amount of hydrogen ions, which changes the pH of water and phosphate-buffered saline significantly within 30 s of treatment [2]. We tested the hypothesis that the e-plasma treatment triggers coagulation through changes in the pH of blood. This was tested by measuring the pH of each blood sample immediately after e-plasma treatment, as described in Section II. Fig. 7 shows that no significant change in pH occurs in the anticoagulated blood samples during the time needed for the e-plasma-treated blood to coagulate.

The changes in pH obtained through e-plasma treatment are smaller than the natural variation of pH found in stored anticoagulated blood, as evident from the data plotted for the untreated samples (0-s treatment). We therefore concluded that coagulation of blood by e-plasma treatment does not occur due to changes in pH or  $\text{Ca}^{2+}$  concentration.

It was previously demonstrated [2] that significant changes occur in blood plasma protein concentrations after treatment by e-plasma of samples from nonanticoagulated and blood with various anticoagulants. Direct activation of intermediate protein factors by e-plasma may be one of the mechanisms of coagulation of blood.

As shown in Fig. 5, conversion of fibrinogen into cross-linked fibrin fibers is the final step in coagulation of blood [29]. Therefore, we postulated that one of the pathways by which e-plasma treatment coagulates blood may be through the direct effect on fibrinogen.

To test this hypothesis, we investigated the effect of the e-plasma treatment (30 s) on a buffered fibrinogen solution (TRIS-buffered saline solution, 20 mM) at a physiological pH of 7.4 using the setup used for blood treatment. As compared to the untreated fibrinogen solution shown in Fig. 8(a), the opacity of the treated fibrinogen solution changes, as shown in Fig. 8(b), indicating that e-plasma initiates changes in the fibrinogen solution. Fig. 9 shows that the pH of the buffered fibrinogen solution over the duration of treatment does not change significantly.

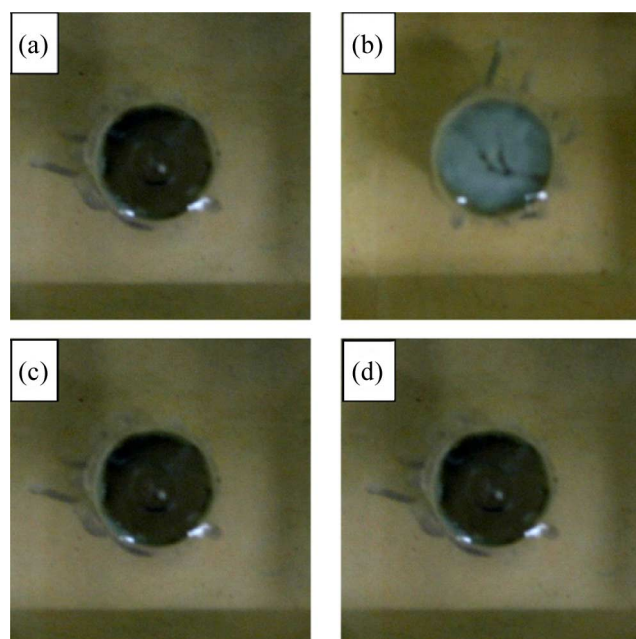


Fig. 8. Treatment of the [(a) (control) and (b) (30 s)] buffered solution of fibrinogen and [(c) (control) and (d) (30 s)] buffered solution of albumin. (a) and (b) There is a change in opacity of the fibrinogen solution after treatment. (c) and (d) There is no change in opacity of the albumin solution.

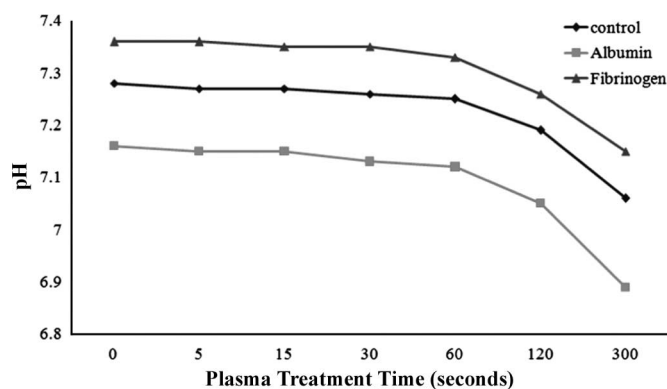


Fig. 9. pH of the buffered solution of albumin and fibrinogen (average error is less than 0.01 unit).

The same solution of fibrinogen in contact with aluminum foil cover exhibits no change in opacity after e-plasma treatment. Thus, the transfer of thermal energy due to e-plasma treatment is not responsible for the observed opacity changes.

DLS measurements on the treated and untreated fibrinogen solutions were carried out to test the possibility that the observed changes in opacity are due to conversion of fibrinogen into larger molecular structures. Fig. 10(a) compares the untreated (control) and treated fibrinogen solutions. The diameter of a fibrinogen molecule is around 9 nm [29], which is demonstrated in Fig. 10(a). The treated fibrinogen exhibits a multimodal distribution of sizes, with the largest increase in size by about two orders of magnitude as compared to the control. The largest fibrinogen aggregates have an average size of 2  $\mu\text{m}$ , which corresponds in the order of magnitude to the size of the fiberlike structures visible in the SEM image [Fig. 3(b)] of the blood clot.

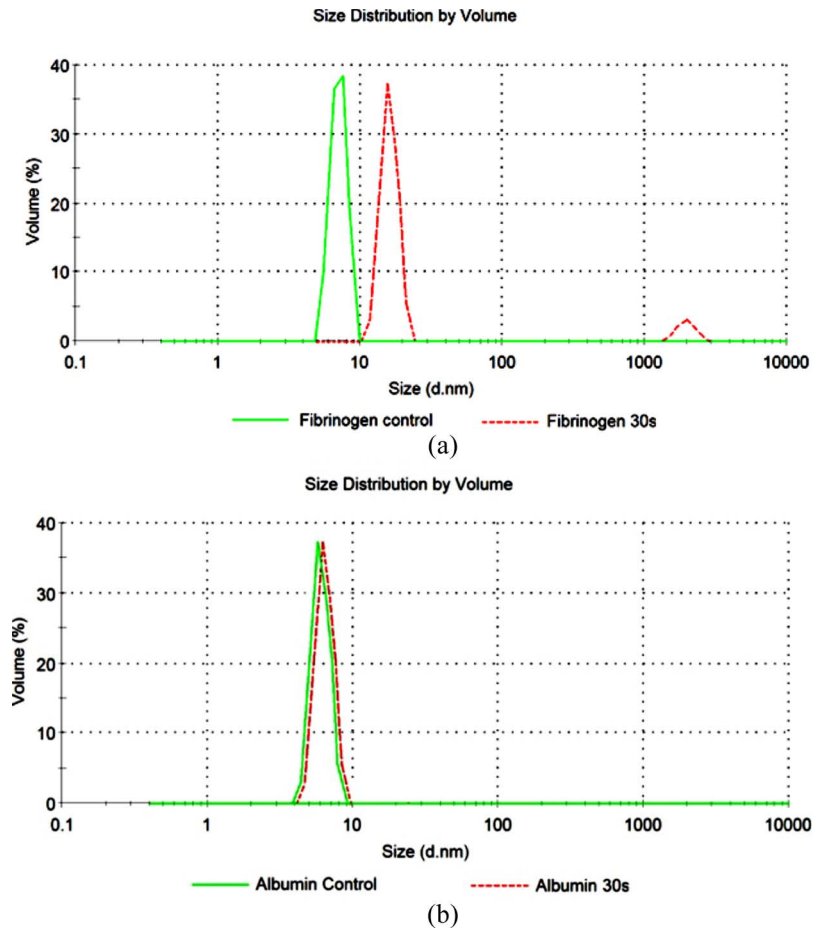


Fig. 10. (a) Comparison of the control and treated solutions of fibrinogen. (b) Comparison of the control and treated solutions of albumin. Treated and untreated solutions of albumin show the same size distribution, with an average size of about 6 nm, which corresponds well to the published albumin size of around 8 nm [30], [31].

The two well-defined peaks in the distribution of sizes of the treated fibrinogen indicate that the fibrinogen conversion is not a nonspecific aggregation that would have resulted in a single broad distribution of aggregate sizes. The aforementioned results, together with the SEM images (Fig. 4) showing formation of fibers, suggest that one important pathway through which e-plasma treatment coagulates blood plasma is the direct conversion of fibrinogen into fibrin fibers. The question arises: How specific is the effect of the e-plasma treatment on the proteins found in blood?

To test the specificity of the e-plasma treatment on blood proteins, albumin solution was tested in the same fashion as the fibrinogen solution. Albumin is an important blood protein that does not participate in the coagulation cascade but is used as a biological glue in some cases [32], [33]. However, the albumin solution that is prepared in the same way as the fibrinogen solution shows no visual change even after 10 min of e-plasma treatment [Fig. 8(c) and (d)]. The pH of the buffered solution of albumin does not change significantly over the duration of shorter treatment, as shown in Fig. 9.

DLS studies of e-plasma-treated albumin solution show no change from the untreated solution, as evident in Fig. 10(b). The fact that albumin is unaffected by the e-plasma treatment indicates that this treatment can be specific in its effects on blood proteins.

#### IV. CONCLUSION

It has been demonstrated earlier that nonthermal plasma coagulates blood rapidly [2], and the results presented in this paper indicate that the nonthermal DBD treatment is capable of coagulating anticoagulated blood. This discharge appears to promote rapid blood coagulation by enhancing the natural coagulation processes. Previously, it was hypothesized that direct contact nonthermal e-plasma treatment initiates blood coagulation by an increase in the concentration of calcium ions [2], an important ion in the coagulation cascade. Our experiments have shown no significant change in calcium concentration during the time of coagulation in the discharge treated blood. E-plasma treatment does not coagulate blood due to the change in pH, as we have observed no significant change in the pH of blood during the time of treatment in which blood coagulates. We hypothesize that e-plasma treatment may activate some of coagulation proteins. E-plasma treatment of a buffered solution of human fibrinogen results in rapid fibrinogen aggregation. Interestingly, this nonthermal plasma treatment is selective, as a similar buffered solution of human serum albumin shows no change even after a longer treatment. The results presented in this paper have indicated that the direct conversion of fibrinogen into fibrin may be one of the mechanisms by which non-thermal plasma initiates coagulation. Further investigations are

necessary to determine other pathways of activation of coagulation by nonthermal plasma treatment.

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