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RESEARCH ARTICLE

PLASMA PROCESSES AND POLYMERS

The active role of organic molecules in the formation of long-lived reactive oxygen and nitrogen species in plasma-treated water solutions

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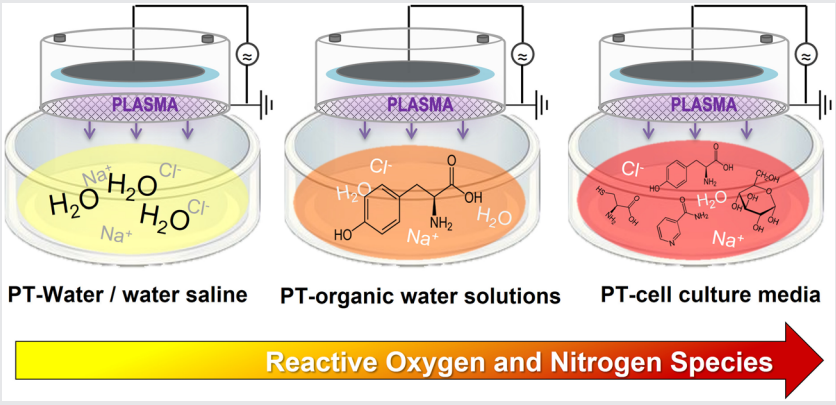
Abstract

Plasma-treated water solutions (PTWS) allow the delivery of reactive oxygen and nitrogen species (RONS) to cells and tissues for different purposes. The mechanism of RONS formation has been clearly modelled in simple liquids like water, by assuming a plasma-driven process independent from the liquid. PTWS for biological experiments, however, are often produced from solutions of complex composition, where the formation mechanism of RONS is far from being understood. In this paper, we describe how water, phosphate-buffered saline solution and two cell culture media were plasma-treated in different conditions to demonstrate how the different composition of the liquids affects the formation of stable RONS (H_2O_2 and NO_2^-) in the resulting PTWS, especially when aromatic organic molecules are present.



Abbreviations: DBD, dielectric barrier discharge; DMEM, Dulbecco's modified Eagle medium; MEM, minimal essential medium; NTP, nonthermal plasma; PBS, phosphate-buffered saline; PT, plasma-treated; PTWS, plasma-treated water solutions; RONS, reactive oxygen and nitrogen species; RPMI, Roswell Park Memorial Institute.

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KEYWORDS
cell culture media, reactive oxygen and nitrogen species, plasma-treated water solutions, plasma medicine, plasma–liquid interactions

1 | INTRODUCTION

Non-thermal plasmas (NTPs), defined as ionized gases in non-equilibrium conditions, offer several technologies, processes and products for medicine and biology, among several other fields.[1] Besides many applications in surface modification processes of biomaterials,[2–7] recently NTPs emerged as newer tools in life sciences for clinical therapies in oncology and wound healing,[8] due to their ability to intervene in the redox control of oxidative diseases like cancer[9,10] or infections.[9,11] Therapeutic effects of NTPs arise from their ability to generate gas admixtures of reactive oxygen and nitrogen species (RONS) in air, close to living matter.[12,13] RONS, well known as natural redox modulators, are involved in the stimulation or inactivation of many cellular functions such as growth, adhesion or death.[14]

Plasma medicine, hence, comes up with the idea to exploit NTP processes to exogenously deliver RONS to living matter to control cellular functions for therapeutic purposes.[9,15,16] It is important to mention that direct NTP treatments are always mediated by the presence of liquids like blood or exudate, water solutions whose components (i.e., inorganic salts, organic molecules and cells) can actively contribute to the further benefits or detrimental effects observed. These effects can be also obtained by using Plasma-Treated Water Solutions (PTWS),[17] namely, water-based liquid media enriched with RONS mixtures by NTPs ignited in N_2/O_2 mixtures with or without the presence of an inert gas like He or Ar. The Biological effects of PTWS are similar to those of direct NTP treatments, and could be used in clinics for wound healing,[18] cancer treatments[19–22] and disinfection.[23–26] Due to the possibility to be administered to various districts of the body, including those not easily accessible to plasma sources, PTWS-based therapies may become even preferred to direct NTP treatments.

The original composition of the liquid and the presence of water vapour have to be considered, to better elucidate the nature of such systems. Reactions with various molecules in the liquid generally extinguish transient species (primary RONS) like $^{\bullet}OH$, O_2^-, $^{\bullet}O$, N_2^* and others to form stable (secondary) RONS like H_2O_2, NO_2^- and NO_3^- in the PTWS.[27–29] These reactions, moreover, dynamically change the chemical composition of the treated liquids in a complex way, which strongly depends on their initial composition.[30,31] In most in vitro experiments, then, the liquid used for RONS delivery must match the conditions for sustaining cell cultures, generally demanding a buffered pH and high concentrations of electrolytes and nutrients (amino acids, vitamins, growth factors, glucose). For these reasons, in simple cases, PTWS are generated from physiological or Phosphate-Buffered Saline (PBS) solutions;[32–34] much more frequently, indeed, cell culture media are preferred,[21,35–38] with Dulbecco's Modified Eagle Medium (DMEM) and Roswell Park Memorial Institute (RPMI) medium among the most commonly used.[39]

The use of cell culture media allow studying at the best the effect of exogenous RONS delivery to cells, as their composition is optimized for culturing cells in vitro. Moreover, their chemical composition is similar to that of biological fluids like blood or exudates present during direct NTP exposure of tissues, whose alteration has never been studied in depth. Typical cell culture media generally, count more than 45 different components in high concentrations (from $mg\,L^{-1}$ to $g\,L^{-1}$) among inorganic (chlorides, metal ions, carbonate or phosphate buffers) and organic molecules (amino acids, vitamins, carbohydrates) plus optional additives like antibiotics and specific growth factors. The presence of these molecules makes the chemical composition of PT-culture media quite hard to be defined after NTP treatments, even though their biological efficacy is clearly evident. The study is also complicated by the need of using different media depending on the specific cell line under investigation.[20,40] Quite frequently, for example, the effect of PTWS exposure is evaluated on many cell types in the same experiment, and the medium used to generate PTWS is changed depending on the cellular model under investigation. As it is often assumed that RONS enrichment in the liquids is exactly the same as long as the same plasma treatment is performed, in several cases the medium used for PTWS is not even specified, as highlighted by E. Biscop et al.[40] This assumption, indeed, is wrong in most cases.

Relevant compositional differences among different cell culture media are found in the concentration of buffer or antioxidant species,[40,41] which are very likely involved in the scavenging of plasma-generated RONS. Therefore, overlooking the composition of the liquid may undermine the validity of in vitro results, as different cell types may be actually exposed to PTWS with a very different chemical composition resulting from the same

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NTP treatment. This aspect, combined with the scarce homogeneity of the NTP sources generally confines the validity of the biological effectiveness of PTWS within the specific lab in which they are produced, or leads to misleading comparisons of cell responses. This could be easily avoided by accurately evaluating the chemical composition of each PTWS, especially when they are generated from different culture media.

Predicting the chemical composition of PT-culture media, though, is hard to achieve. The complexity of the specific plasma–liquid interactions, in fact, must be added to the complex panorama of chemical reactions in the plasma phase. Most of the modelling studies concerning the mechanism of RONS formation in PTWS have been performed in liquids of simple composition like water,[25,26,28,28,42,43] by assuming plasma-driven processes supposed valid also in other liquids. In contrast, recent evidence attest that the formation of RONS in PTWS is heavily affected by the initial composition of the liquid. Regarding $^{\bullet}NO$, for example, we have recently reported that, after the same plasma treatment, $^{\bullet}NO$ was revealed in the water while it was not in DMEM, partly for its scavenging by organic compounds in the medium and partly for the inhibition of formation routes at the buffered neutral pH of the medium.[44] In the same direction, Yan et al.[45] have found that H_2O_2 plasma-produced in DMEM was consumed, during storage, by reactions with specific amino acids such as cysteine and methionine, while the consumption was prevented by adding 3-nitro-tyrosine in DMEM, without specifying the 'protection' mechanism exerted by these molecules toward H_2O_2.[45] Privat-Maldonado et al.,[46] instead, reported the overproduction of long-lived RONS like H_2O_2 and NO_2^- in PT-cell culture media with respect to PT-water and PT-PBS after the same plasma treatment.[46] It is quite clear, thus, that biomolecules may affect the final RONS distribution in PTWS not only through scavenging processes but also through the promotion/inhibition of specific RONS production pathways in the liquid phase. In this paper, this last aspect is extensively discussed.

For this purpose, we have monitored the amounts of H_2O_2 and NO_2^- in PTWS generated from four liquids with very different chemical compositions: water, PBS and two cell culture media, DMEM and RPMI. Each liquid was exposed to Dielectric Barrier Discharges (DBDs) ignited in a wide range of operating conditions, by changing feed gas composition, treatment time and plasma energy dose. The contribution of each phase, the liquid and the plasma, to RONS generation, is analyzed and discussed. The data reported demonstrate that specific biomolecules in cell culture media effectively create alternative routes of RONS formation, which cannot be related to the action of plasma alone, above all in the case of H_2O_2. We, therefore, consider the results reported here of extreme importance for understanding the dynamic evolution of the chemical composition of PTWS. We propose to optimize the composition of each single PTWS for each specific biological protocol to be used in experiments and in therapies.

2 | EXPERIMENTAL SECTION

2.1 | PTWS generation

PTWS were generated with a DBD PetriPlas$^+$ plasma source engineered by the Leibniz Institute for Plasma Science and Technologies (INP), and properly modified by E. Sardella in collaboration with INP researchers. A schematic overview of the apparatus is reported in Figure 1. The source consists of a Plexiglas flow unit, set with gas connections, and a discharge unit. This latter is composed of a stainless-steel ground grid 4 mm far from a disc-shaped high-voltage (HV) copper electrode (3 cm dia), covered with a glass dielectric disc (1 mm thick). A dry diaphragm pump (Pfeiffer Vacuum; Aßlar) is connected to the flow unit to evacuate the exhaust and keep the pressure constant at 760 Torr, as measured with an MKS Baratron. An electric field (6-kHz frequency) at different applied peak-to-peak voltages (V_{pp}) was generated with a power supply connected to a programmable 10 MHz DDS function generator (TG1010A; Aim-TTi) to ignite a volume discharge as wide as the HV electrode. The discharges were pulsed with a 25% duty cycle (DC; 25 ms plasma on, t_{on}) over a period ($t = t_{on} + t_{off}$) of 100 ms.

The discharge unit is properly adapted for the remote treatment of liquids in commercial TPP® Petri dishes (57 mm dia, Techno Plastic Products; Trasadingen) housed under the reactor by means of a slot of the same diameter on the flow unit. When the dish is positioned, the gap between the liquid and the discharge becomes a closed system; purging the gap with the gas feed before igniting the plasma allows controlling the chemical composition in the gap. The treatment is achieved by the diffusion of RONS from the plasma to the liquid through the grid. Pure O_2 (air liquid, 99.999%), N_2 (air liquid, 99.999%), synthetic air (air liquid, 99.999%) and different O_2/N_2 mixtures were used as feed.

The gas flow rates were controlled with MKS mass flow controllers and kept constant at 0.5 L min^{-1}.

The influence of the operating conditions thought to impact RONS concentrations in PTWS was extensively analyzed; treatment time, gas feed, applied voltage, treated liquid volume and treatment distance were varied

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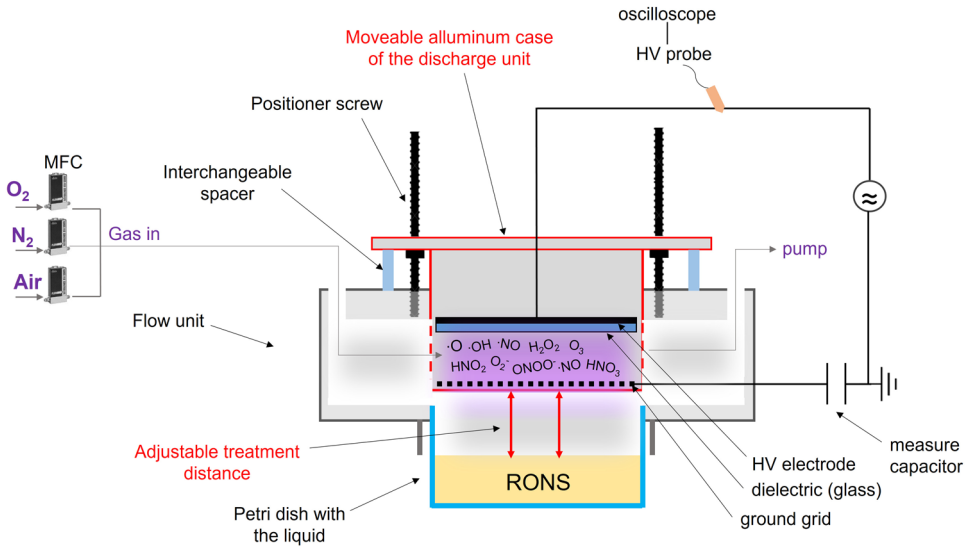

FIGURE 1 Experimental setup to treat the liquids: Schematic overview of the plasma source. HV, high-voltage; MFC, mass flow controller; RONS, reactive oxygen and nitrogen species

TABLE 1 Parameters changed to tune the amount of H_2O_2 and NO_2^- in PTWS

Parameter	Variation
Liquid	DMEM, RPMI, PBS, Water
Gas feed	O_2, N_2, O_2/N_2, synthetic Air
Treatment time	60–300 s
Applied voltage (V_{pp})	10–14 kV
Treated volume	2–5 ml
Treatment distance	2–5 mm

Abbreviations: DMEM, Dulbecco's modified Eagle medium; PBS, phosphate-buffered saline; PTWS, plasma-treated water solution; RPMI, Roswell Park Memorial Institute.

to investigate the influence of each parameter on RONS concentration in the resulting PTWS. The ranges of each parameter are reported in Table 1. Detailed experimental conditions are reported in Section 3.

The discharge unit is housed in the Plexiglas flow unit with metallic positioner screws (Figure 1) designed to change the distance of the discharge unit from the surface of the liquid, regardless of the volume of liquid. This approach was used in the series of discharges at variable liquid volume (2–5 ml), and at a variable distance (2–5 mm) with a fixed liquid volume (2 ml).

The generation of RONS was analyzed in four different liquids: double-distilled water, PBS (cat. N: P3813; Sigma-Aldrich), DMEM (cat. N. D1145; Sigma-Aldrich) and RPMI (cat. N. R7509; Sigma-Aldrich). In some of the experiments, plasma treatments were performed also in L-cysteine (cat. N. C8755; Sigma-Aldrich) or L-tyrosine (cat. N. T-3754; Sigma-Aldrich) solutions, and in a minimum essential medium (MEM) amino acids solution (cat. N. 11140050; Gibco®, Thermo Fischer Scientific), containing the same non-essential amino acids of the standard MEM. The MEM amino acids solution was diluted 1:100 with water. Cell culture media were used without phenol red, to exclude spectroscopic UV-Vis interferences during the colorimetric detection of RONS, and without any of the supplements (i.e., fetal bovine serum or antibiotics) used in cell culture routines. The pH of the liquids was measured with a pH meter (HI8424; Hanna instruments).

2.2 | Electrical characterization of the plasma

The electrical characterization of the DBDs was performed by measuring the energy dissipated per each voltage cycle. Instantaneous voltages (ΔV) and charges (Q) were measured, respectively, with an HV probe (P6915A HV probe; Tektronix) and a 100-nF capacitor probe (Cmeas), connected in series to the ground electrode of the DBD and to a digital oscilloscope (TDS 2014 C; Tektronix). The energy dissipated per each voltage cycle was calculated by using the voltage-charge Lissajous method.[47] This energy was multiplied by the number of voltage cycles in one pulse and by the number of pulses in the treatment time, then divided by the area of the copper electrode (7.1 cm^2) to calculate the energy dose delivered in each discharge. At least three

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measurements per DBD were performed per each set of conditions.

2.3 | Detection of H_2O_2 and NO_2^- in PTWS

The detection of H_2O_2 and NO_2^- was carried with colorimetric assays of commercial test kits. A copper-phenantroline assay (cat. N. 118789; Merck Millipore) was used to detect H_2O_2, while the Griess assay (cat. N. 1.14776.001; Merck Millipore) was used to detect NO_2^- ions. Both assays were specifically validated in the case of cell culture media, as shown in our previous paper.[48] UV-Vis absorbance measurements were performed with a Cary 60 UV-Vis spectrophotometer (Agilent) in the spectral range 200–800 nm with 5 nm resolution. Disposable plastic cuvettes (cat. N. 223-9955; Bio-Rad) with a volume of 1 ml and 10 mm optical length were used. Further information about the validation of each assay in the specific case of cell culture media and PTWS can be found in our reference [48].

2.4 | Statistical analysis and data processing

Statistically significant differences among data were computed with the software GraphPad Prism version 6.1. A one-way analysis of variance (ANOVA) was performed with a subsequent Turkey's multiple comparison test by assuming a normal distribution of the data. Statistical results of measurements were shown as the mean value ± standard deviation. All p values <0.01 were considered statistically significant.

2.5 | Fourier-tranform infrared spectroscopy (FT-IR) on PTWS

FT-IR was used to inspect structural modifications of L-cysteine. Plasma treatments were performed on 2 ml L-cysteine solution (100 mg/L) in the same conditions used for the other PTWS (13 kV, 6 kHz, 25% DC, 0.5 slm air, 5 min); 500 µl of the treated solution were left drying overnight, under the hood, on clean shards of Si wafers (1.5 cm × 1.5 cm), at room temperature. FT-IR spectra (32 scans per analysis, 4 cm^{-1} resolution) were acquired in transmission mode with a Vertex 70 V Bruker spectrometer (Thermo Fisher Scientific). The spectrometer was evacuated to less than 150 Pa for 10 min before each acquisition. Spectra were elaborated with Bruker OPUS software.

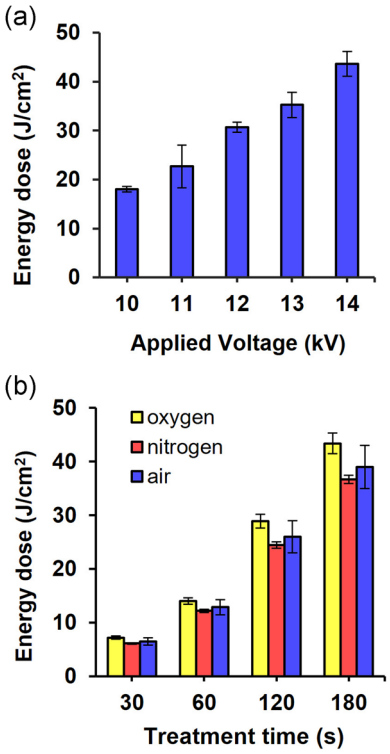

FIGURE 2 Calculated plasma energy dose as a function (a) of applied voltage in a 180 s air DBD; and (b) of treatment time and gas feed in a 13 kV DBD

3 | RESULTS AND DISCUSSION

3.1 | Electrical characterization of the DBDs

The DBD used in the experiments operates in a filamentary regime, with the formation of micro discharges during each voltage cycle, typical for a planar DBD.[49] The Lissajous figures obtained consist of closed parallelograms, as expected for capacitive systems.[50] By measuring the area of the Lissajous figure it is possible to calculate the energy dose of the plasma; as expected, this increases, with applied voltage (Figure 2a) and with treatment time (Figure 2b). The mean energy dose for an air DBD ignited at 13 kV for 180 s is 39 4 J/cm^2. In Figure 2b, it is also shown that the energy dose is almost the same for O_2, N_2 and synthetic air.

3.2 | pH measurements of the liquids

The dissociation in the liquid of plasma-produced acid species like nitrous and nitric acids leads to nitrite and nitrate ions in PTWS, but also to the formation of H^+ ions. This acidification could potentially affect the formation or the stability of other RONS, or the biological

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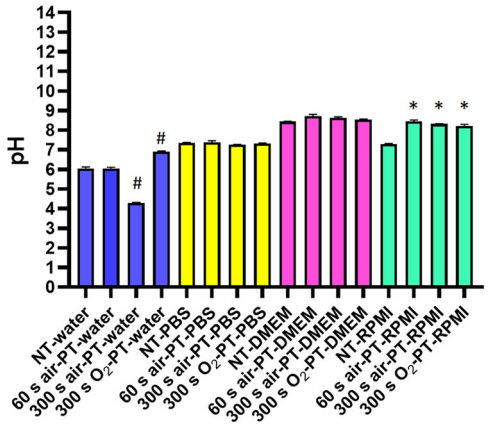

FIGURE 3 pH measurements in water, PBS, DMEM and RPMI before and after a mild (60 s, air DBD) and two harsh (300 s, air and O_2 DBD). All DBDs were ignited at 13.5 kV, 6 kHz, 25% DC, 0.5 slm, on 2 ml of each liquid, with 3 mm of treatment distance. DBD, dielectric barrier discharge; DC, duty cycle; DMEM, Dulbecco's modified Eagle medium; RPMI, Roswell Park Memorial Institute. One-way analysis of variance with Tukey's post-test: $^{\#}p < 0.01$ versus NT-water; $^{*}p < 0.01$ versus NT-RPMI

responses of living matter exposed to these liquids.[51] Therefore, it is important to monitor pH variations, above all when comparing different PTWS generated from buffered and non-buffered liquids. Only water is non-buffered, among the liquids under investigation, while PBS, DMEM and RPMI are buffered to neutral pH. Figure 3 shows the results of pH measurements of all tested liquids, before and after a mild (60 s, air plasma) and two harsh treatments (300 s, air and O_2 DBDs).

As it is shown in Figure 3, in PBS and DMEM no significant alterations of pH were found, not even in the harsher condition. In the case of RPMI, a very small increase of pH was observed after plasma exposure. In the case of water, the milder treatment (60 s, air DBD) resulted in no pH variation, while prolonging the treatment to 300 s resulted in pH values around 4. No acidification was found in water exposed to DBDs fed with pure O_2.

3.3 | Analysis of NO_2^- in PTWS

The results of NO_2^- detection in water, PBS, DMEM and RPMI exposed to DBDs in different operating conditions are reported in Figure 4. The data demonstrate that the NO_2^- amount in PTWS strongly depends on the gas feed (Figure 4a), the treatment time (Figure 4b,c), the applied voltage (Figure 4d) and the volume of the treated liquid (Figure 4e). The values of NO_2^- concentration after the same plasma treatment were found different from one liquid to another. In particular, NO_2^- concentrations in PT-DMEM and PT-RPMI were found similar, but much higher than in PT-PBS and PT-water, as shown in each data series of Figure 4, especially when the $O_2/(O_2 + N_2)$ ratio is lower than 0.5. However, similar NO_2^- trends were found in each as a function of the plasma parameters. In all untreated liquids, the concentration of NO_2^- was below the detection limit.

The results here reported are coherent with the mechanism for NO_2^- production in PTWS reported in Figure 5. According to the literature, the main precursor of NO_2^- is gaseous HNO_2, formed mostly in the plasma phase during $^{\bullet}NO$ oxidation by $^{\bullet}OH$ radicals (reaction 1 in Figure 5). When O_2 and N_2 are present in the feed, in fact, $^{\bullet}NO$ is generated through the well known Zeldovich mechanism,[43] and it can also be detected in PT-water.[44] Moreover, further oxidation of $^{\bullet}NO$ by O_3 or O_2 (reaction 2 and 3) can generate $^{\bullet}NO_2$ in the plasma phase that reacts with water molecules, as vapour or in the liquid, to produce HNO_2 as well (reaction 5 in Figure 5), that dissolves and dissociates in water to form NO_2^- ions, as reported in reaction 6 of Figure 5.

In agreement with the assumed mechanisms, hence, the production of NO_2^- ions appears strongly dependent on $^{\bullet}NO$ and its derivatives in the plasma, whose formation in presence of N_2 and O_2 was assessed in our previous work.[44] In fact, the NO_2^- concentration depends on the N_2 content in the feed (Figure 4a): N_2-rich plasmas ($O_2/O_2 + N_2 < 0.4$) maximize the amount of NO_2^-, while O_2-rich plasmas minimize it. By working in O_2 feed, in absence of N_2, NO_2^- ions are practically absent in all PTWS (<20 μM) under investigation and no acidification of the liquids is found. This demonstrates that N_2 is the limiting agent for the formation of nitrites. This trend is observed in all investigated liquids.

In parallel, an increase of the plasma energy dose, by raising the treatment time from 60 to 300 s (Figure 2d) or the applied voltage from 10 to 15 kV (Figure 2c), excites a greater number of N_2 molecules in the plasma, which result in greater amounts of NO_2^- in the liquids (data in Figure 4b,d). Clearly, in absence of N_2, increasing the energy dose generates only minimal NO_2^- amounts (<20 μM) in the liquids probably due to the residual air in the system (Figure 4c).

The above-mentioned considerations, though, do not account for the production of nitrite ions when no O_2 is in the system (Figure 4a). When pure N_2 is used in a closed DBD like ours, relevant O_2 contaminations are quite unlikely. In case of a not well-sealed system, we would have observed relevant N_2 contamination in O_2 DBDs; with this feed, instead, we could not find NO_2^- in the liquids, attesting for the absence of air leaks. The only way to form nitrites or even NO would be to provide a source of oxygen in the plasma. As shown in Figure 4a, the optimal feed composition to form NO_2^- in the liquid

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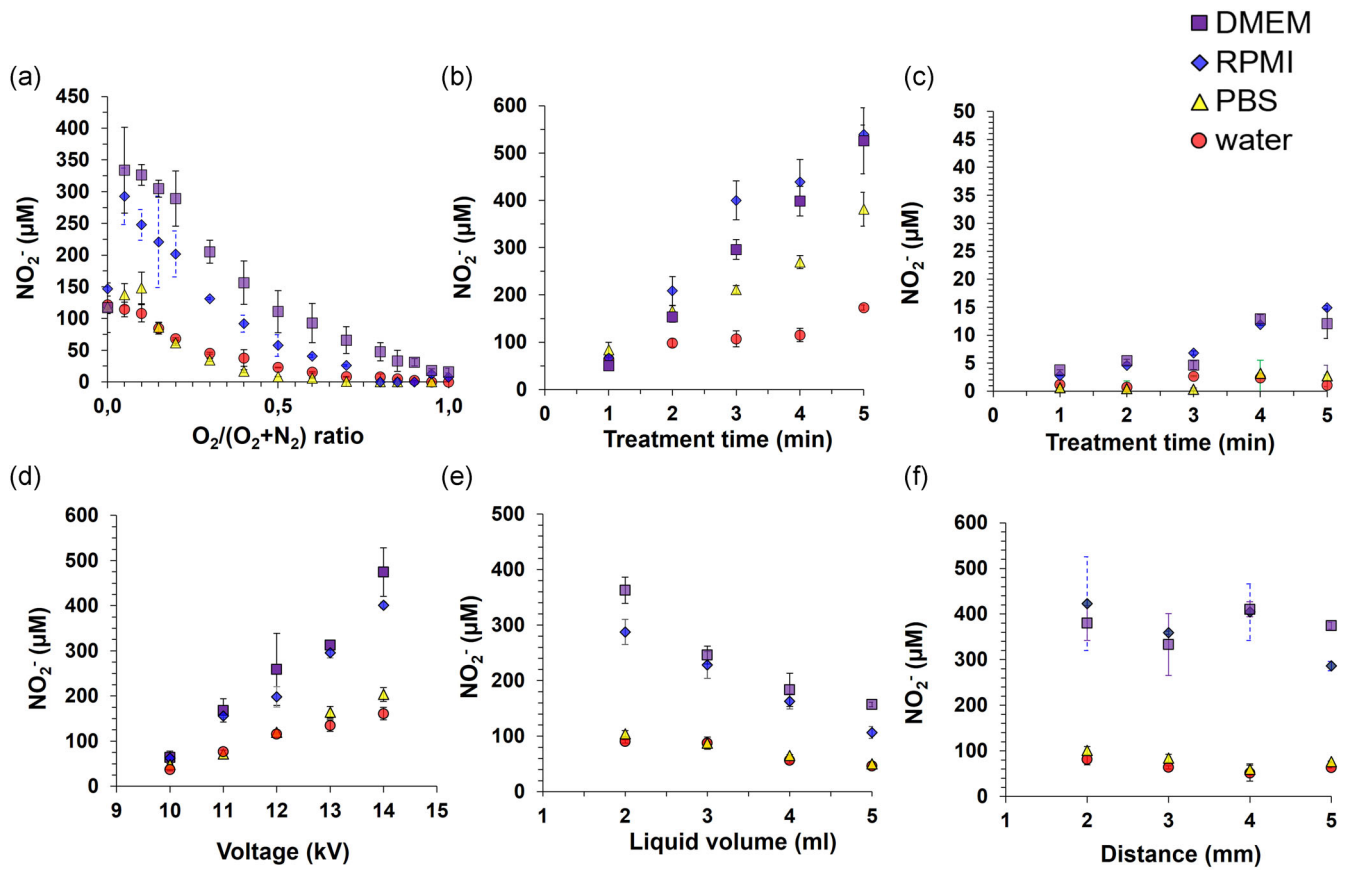

FIGURE 4 NO_2^- concentrations in PT-water, -PBS, -DMEM and -RPMI as a function of (a) $O_2/O_2 + N_2$ feed ratio; and treatment time in (b) air and (c) O_2; (d) applied voltage; (e) liquid volume; (f) treatment distance.
Unless differently specified, all DBDs were ignited in air (0.5 slm) at 13 kV, 3 mm treatment distance, 2 ml of liquid, for 180 s. DBD, dielectric barrier discharge; DMEM, Dulbecco's modified Eagle medium; PT, plasma-treated; RPMI, Roswell Park Memorial Institute

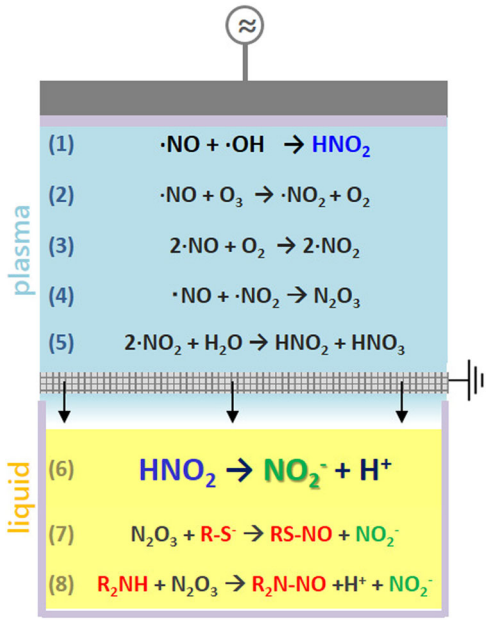

FIGURE 5 List of reactions in plasma and liquid phase involved in the formation of NO_2^- ions[31,43,44,52,52,53]

is 95% N_2 and 5% O_2. This means that even small quantities of O_2 promote the formation of NO_2^-. In pure N_2, traces of O_2 can be introduced by residual air, and by water evaporated from the liquid.

By fixing treatment times, applied voltage and feed composition, in principle, the amount of HNO_2 produced in the plasma should be constant. Accordingly, the volume increase of the liquids from 2 to 5 ml dilutes HNO_2 dissolved and, consequently, decreases the concentration of NO_2^- (Figure 4e). On the contrary, an increase in the treatment distance from 2 to 5 mm does not affect the amount of NO_2^- ions. Therefore, we can conclude that the NO_2^- formation in PTWS, in our conditions, is related to the presence of RNS, $^{\bullet}NO$ or $^{\bullet}NO_2$, in the plasma phase. Accordingly, the amount of NO_2^- in the liquids can be successfully optimized by properly tuning plasma feed gas composition, applied voltage and treatment time.

In spite of this, the concentration of NO_2^- ions in PT-cell culture media was found higher than in PT-PBS or PT-water, in each series of Figure 4, after the same

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plasma treatment. This difference should be related to the different chemical compositions of the liquids. The presence of inorganic salts (NaCl, KCl and others) is not responsible for the overproduction of NO_2^- in the culture media, as these species are contained in similar concentrations also in PBS. Instead, cell culture media contain high concentrations of organic molecules, which include amino acids, vitamins and carbohydrates. Our hypothesis, in fact, is that these molecules can be involved in nitrosation reactions with transient RNS like NO, NO_2 or N_2O_3 to form nitro-organic molecules that could release additional NO_2^- ions.

Nitrosation reactions generally involve electrophilic species like NO^+ or NO, capable of reacting with electron donors through the nitrogen atom.[54] In cell culture media with neutral pH, however, the possibility to find NO or NO^+ is negligible because they persist only in strong acid conditions.[54] Nonetheless, it is possible that the nitrosation of the biomolecules involves NO^+ donor species like N_2O_3, which can be formed by plasma-generated NO and NO_2, and dissolves in aqueous media (reaction 4, in Figure 5),[23] with the consequent release of NO_2^- ions. Thiol groups of sulfur-containing amino acids[44,53–56] like cysteine and methionine, or the amino groups[54,57] on side chains of many amino acids may be targeted by N_2O_3, according to reactions 7 and 8 in Figure 5, effectively releasing nitrite ions in the media.

Demonstrating this chemical mechanism, however, would require specific kinetics experiments. We have previously investigated the production of •NO in PT-water and in PT-DMEM and confirmed that, while •NO was detected in PT-water, it was absent in PT-DMEM due to the scavenging action of some organic compounds (D-glucose in prevalence) and to the buffered pH, far from the acid conditions needed for •NO formation in liquids.[44] Moreover, several studies report thiols nitration in L-cysteine and L-methionine to form S-nitroso-organic compounds in plasma-treated liquids.[44,53,55,56]

For this reason, we have used FT-IR spectroscopy to record the chemical fingerprint of an L-cysteine solution after an air DBD to investigate the structural modification of the biomolecule. The treatment was performed on 2 ml of cysteine solution in water (100 mg/L), not acidified with HCl, in the same conditions used for the other PTWS (13 kV, 6 kHz, 25% DC, 0.5 slm air, 5 min). 500 µl of the treated solution were left drying overnight, under the hood, on Si shards (1.5 × 1.5 cm), at room temperature. The FT-IR spectra of untreated (NT-Cys) and plasma-treated cysteine solution (PT-Cys) are shown in Figure 6.

Structural changes in PT-Cys include (i) oxidation of -SH groups to -S═O (ν_{SO} at 1040 cm^{-1}); (ii) formation

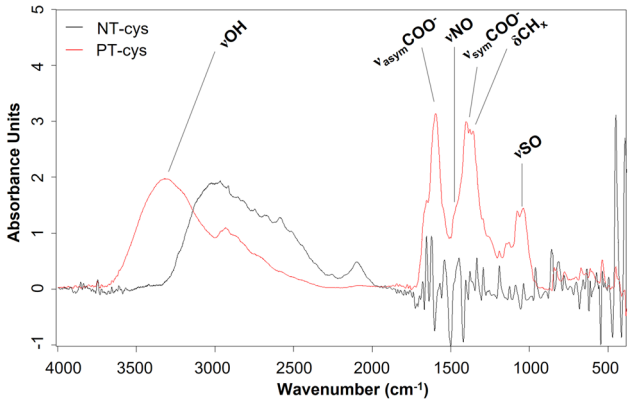

FIGURE 6 FT-IR spectra of untreated (NT-cys, in black), and plasma-treated cysteine solution (PT-cys, in red)

of -OH groups (ν_{OH}, 3200–3500 cm^{-1}); (iii) formation of -COOH groups ($\nu_{asymCOOH}$, 1610 cm^{-1}); (iv) nitrosation of -SH group (resonance structure of S-nitrosothiol group, $\nu_{N=O}$, 1487 cm^{-1}). It is possible that oxidation and nitrosation of -SH group may occur at the same time, on different cysteine molecules in the solution. In this way, the nitrosation of the thiol groups may effectively be a possible secondary route for NO_2^- production in culture media. Although FT-IR spectra support this hypothesis this last aspect needs to be fully elucidated.

3.4 | Analysis of H_2O_2 in PTWS

The results of H_2O_2 detection in DMEM, RPMI, PBS and water exposed to different DBDs are reported in Figure 7. Unlike the production of NO_2^- ions, the data indicate that the production of H_2O_2 depends very strongly on the type of liquid treated rather than on the plasma conditions varied. Different trends are observed between the two cell culture media and water or PBS.

In particular, it is observed that in our conditions the formation of H_2O_2 in water and in PBS is inhibited (<10 µM, the limit of detection of the colorimetric assay[48]) in all conditions, despite the wide range of parameters tested (Figure 7). On the contrary, in the two culture media, the production of H_2O_2 is observed, with more scattered trends as a function of the individual plasma parameters with respect to NO_2^-. Moreover, the concentration of H_2O_2 in PT-DMEM was found higher than in PT-RPMI after the same treatments, in all cases, as it clearly emerges in Figure 7. In both culture media, an increase in the H_2O_2 amount is observed by increasing the $O_2/O_2 + N_2$ ratio (Figure 7a), the treatment time (Figure 7b,c) and the applied voltage (Figure 7d). On the contrary, it remains almost constant by increasing liquid volume (Figure 7e) and treatment distance (Figure 7f).

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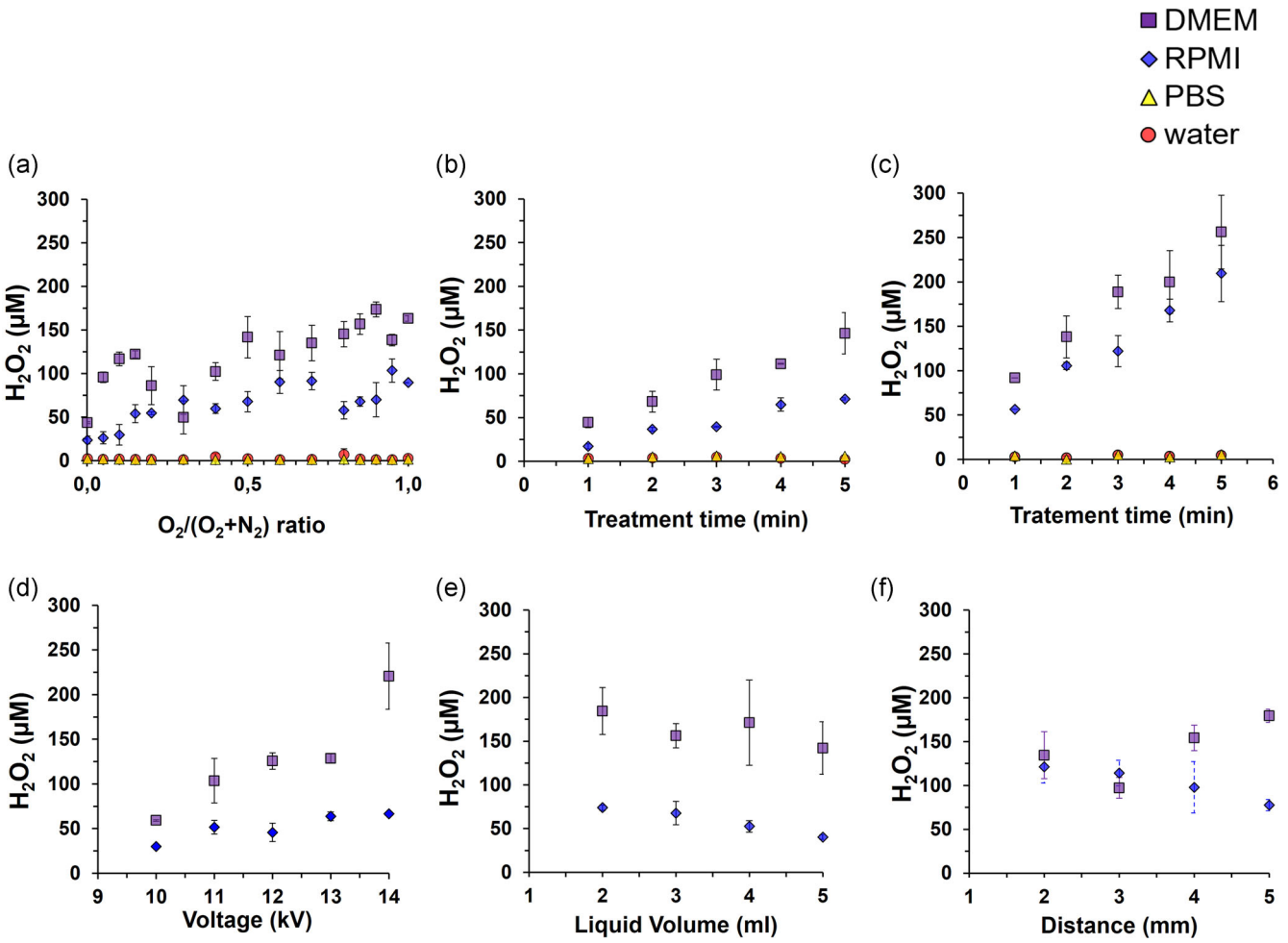

FIGURE 7 H_2O_2 concentrations in PT-water, -PBS, -DMEM and -RPMI as a function of (a) $O_2/O_2 + N_2$ feed ratio; treatment time in (b) air and (c) O_2; (d) applied voltage; (e) liquid volume; (f) treatment distance.
Unless differently specified, all DBDs were ignited in the air (0.5 slm) at 13 kV, 3 mm treatment distance, far from 2 ml of liquid, for 180 s.
DBD, dielectric barrier discharge; DMEM, Dulbecco's modified Eagle medium; PT, plasma-treated; RPMI, Roswell Park Memorial Institute

Indeed, the results here reported strongly challenge the mechanism of H_2O_2 production in PTWS assumed so far in the literature, schematically reported in Figure 8. It is generally mentioned that the main precursor of H_2O_2 in liquid is the $^{\bullet}OH$ radical,[26,27,42,42,58–60] which is formed in the plasma from H_2O molecules by dissociation through electron collisions (reaction 1), photodissociation (reaction 2), or its reaction with $^{\bullet}O$ atoms (reaction 3).[52] The formation of H_2O_2 is mostly attributed to $^{\bullet}OH$ recombination in the plasma (reaction 8, in presence of a collision partner M) or in the aqueous phase (reaction 9), where $^{\bullet}OH$ is highly soluble.[52] Moreover, also the hydroperoxyl radical $HO_2^{\bullet}$ (reaction 4) can react with $^{\bullet}H$ (reaction 5) or with $^{\bullet}O$ (reaction 6) atoms to restore $^{\bullet}OH$, or it can recombine to form H_2O_2 (reaction 7). The occurrence of such a mechanism is clearly related to the presence of water vapour molecules in the plasma phase to produce $^{\bullet}OH$ or $HO_2^{\bullet}$ radicals.

Despite this, however, we could not find H_2O_2 in PT-water or PT-PBS, while it was detected in high concentration in both PT-culture media. Indeed, the organic molecules and the buffered pH in these liquids should quench the oxidative capacity of RONS and reduce their concentration.[44] Many groups, though, have recently reported that organic compounds increase rather than decrease the production of RONS in PTWS. For example, Privat-Maldonando et al.[46] reported that the lack of bactericidal activity in plasma-treated solutions with high organic content, like DMEM and other culture media, is correlated with the decrease of transient RONS, such as $^{\bullet}OH$, O_3 or $^{\bullet}O$ and with the increase of H_2O_2 and NO_2^- (with lower bactericidal effect).[46] Similarly, also Ranieri et al.[59] have found long-lived RONS more concentrated in PT-RPMI than in PT-water after the same treatment, and concluded that reactions between medium components and plasma-produced species may have led to the

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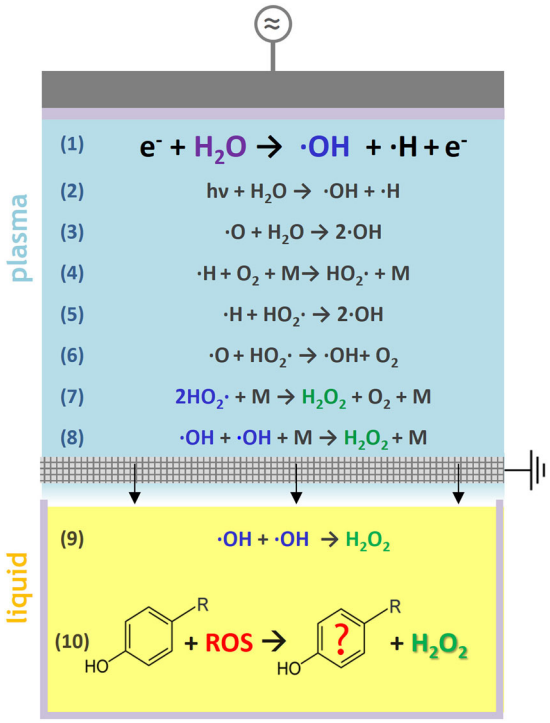

FIGURE 8 List of reactions in plasma and liquid phase involved in the formation of H_2O_2 in treated liquids[27,42,52,58,59]

overproduction of long-lived RONS in RPMI.[59] Concerning this topic, Kurake et al.[55] specifically questioned the role of ˙OH recombination to form H_2O_2 in PT-cell culture media, and concluded that ˙OH radical may more likely oxidize aqueous organic molecules instead of directly generating H_2O_2.[55] In line with these findings, it is our opinion that ˙OH recombination has a minimal role in the production of H_2O_2 in PTWS is our conditions. More likely, H_2O_2 is formed, indeed, in still unknown secondary reactions involving organic molecules in the liquid.

As shown in the reactions in Figure 8, the presence of water molecules in the plasma phase is required to generate the ˙OH, ˙H and HO_2˙ species involved in H_2O_2 formation. During the experiments, dry gases (O_2, N_2 and air) were used to purge the plasma–liquid headspace and to ignite the DBDs. Hence, in our system, water molecules can only come from evaporation. Since treatment times are short (till 300 s), the plasma is not in contact with the liquid and its temperature remains constant during the treatment, this contribution can be considered negligible.

This aspect has been clearly described also by Hefny et al.,[61] which reported that H_2O_2 formation in deionized water required the addition of water vapour into the gas feed, while in presence of organic molecules like phenol it was found independent of the humidity of the plasma phase.[61] In effect, the same paper shows that the H_2O_2 formation in PT-phenol solutions is strongly correlated to the presence of ˙O and O_3 species in the plasma, and could proceed also in absence of water vapour. In agreement with these findings, we have found the H_2O_2 formation is stronger in cell culture media exposed to O_2 DBDs (Figure 7c) rather than to air ones (Figure 7b), due to the consumption of oxygenated species to form ˙NO, when N_2 is present in the feed. In the case of PT-cell culture media, therefore, it is our opinion that the production mechanism of H_2O_2 should involve the oxidation of organic molecules in the culture medium. In effect, the oxidation of specific amino acids like tryptophan in the presence of singlet oxygen has been already associated with O_3 and H_2O_2 release in water media.[62,63]

We have tried to better ascertain the nature of the organic molecules involved in the process. The overproduction of H_2O_2 found in PT-DMEM compared to PT-RPMI is a valuable starting point to shorten the list of possible candidates in a mixture counting more than 45 different organic compounds. By comparing the composition of the two cell culture media, we found that both liquids contain a mixture of the same amino acids and vitamins, at different concentrations. Figure 9a shows the concentration ratio of the same organic species present in the two media. It can be seen here that the organic species in DMEM are on average 3.3 times more concentrated than in RPMI, further corroborating their correlation with the higher production of H_2O_2 detected in PT-DMEM.

Due to the large variety of molecules in these media, we have focused our attention on a limited list of compounds, selected for the presence of particular functional groups or structural elements with higher sensitivity to oxidation, such as aromatic and thiol side chains on some amino acids.[30,55,56] Moreover, many species with aromatic moieties are more concentrated in DMEM than in RPMI (listed in Figure 9a), such as L-phenylalanine, L-tryptophan and L-tyrosine, folic acid, pyridoxine, niacinamide and thiamine. In light of these considerations, we have plasma-treated solutions of the amino acids L-tyrosine, representative molecule for aromatic organic compounds, and L-cysteine, because of the presence of a thiol group, whose oxidation to form sulphenyl, sulphinyl and sulphonyl functionalities is known. In parallel, we have also tested a solution of the seven amino acids contained in MEM (glycine, L-alanine, L-asparagine, L-aspartic acid, L-glutamic acid, L-proline, L-serine), each with linear side chains with many other functional groups (amidic, alcoholic, carboxylic and aliphatic), possibly contributing to the formation of H_2O_2.

As shown in Figure 9b, the H_2O_2 concentration in PTWS is clearly related to the presence of L-tyrosine,

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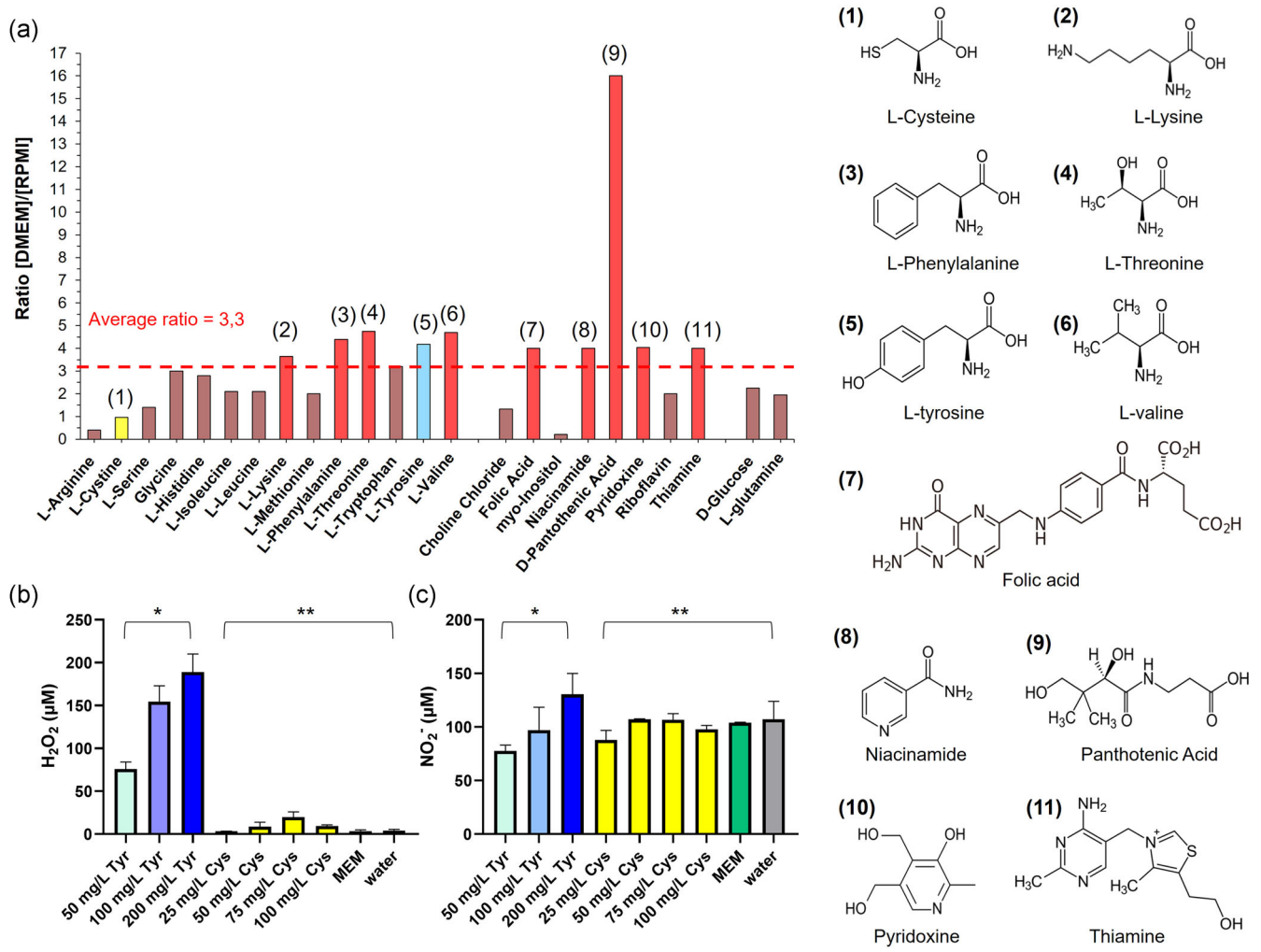

FIGURE 9 (a) Concentration ratio of the organic molecules in the commercial composition of DMEM and RPMI; (b) H_2O_2 and NO_2^- concentrations detected in PT-water, PT-L-tyrosine (50–200 mg/L), PT-L-cysteine (25–100 mg/L) and PT-MEM solutions after an air DBD ignited at 13 kV, 3 mm far from 2 ml of each solution. DBD, dielectric barrier discharge; DMEM, Dulbecco's modified Eagle medium; PT, plasma-treated; PT-MEM, plasma-treated minimal essential medium; RPMI, Roswell Park Memorial Institute. One-way analysis of variance with Tukey's post-test: *$p < 0.05$; **$p > 0.05$

while it is absent in the other two solutions. In the case of L-tyrosine, moreover, the H_2O_2 amount in the liquid is also dependent on its concentration. These results, therefore, confirm that the production of H_2O_2 must involve plasma-induced oxidation of aromatic rings on organic molecules of cell culture media, at least in our conditions. The concentrations of NO_2^- formed in presence of the organic molecules, instead, were found very similar to that formed in PT-water, as shown in Figure 9c. In the specific case of L-tyrosine, however, the concentration of NO_2^- was found slightly dependent on that of the amino acid in the solution, as the amount of nitrite ions formed in a 200 mg/L L-tyrosine solution was significantly higher ($p < 0.05$) than that formed in a 50 mg/L solution. This last result also suggests possible involvement of L-tyrosine in the formation of NO_2^-, as assumed in Section 3.3. From the reported data, we can therefore summarize that the production of H_2O_2 in PTWS, in our conditions, is likely to occur through oxidation reactions of organic compounds with a phenolic ring due to transient ROS like $^{\bullet}O$ and O_3, as schematized in reaction 10 of Figure 7. Concerning the products of tyrosine oxidation, Lukes et al.[25] show that the treatment of a water solution of phenol leads to hydroxylation and ring oxidation reactions forming more than 15 different by-products, including catechol, hydroquinone, 1,4-benzoquinone and hydroxy-1,4-benzoquinone and muconic acid. The extent and the nature of these reactions on tyrosine in our conditions is a current object of our research. We believe that their full understanding is of fundamental importance for the plasma medicine community to finely optimize the chemical composition

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of PTWS of different origins for in vivo and clinical applications.

4 | CONCLUSIONS

We have described the production of long-lived RONS, H_2O_2 and NO_2^-, in water, PBS, DMEM and RPMI after different DBD treatments. We could demonstrate that the presence of specific biomolecules in the cell culture media, like the amino acid L-tyrosine, effectively creates alternative routes of RONS formation, which cannot be related only to the action of the plasma. This effect was much more prominent in the case of H_2O_2 with respect to NO_2^-. By working with a closed system with a highly controllable feed composition, we could exclude external humidity from the discharges and directly observe that, in absence of water vapour in the feed, H_2O_2 could not be formed in PT-water and PT-PBS in any tested condition, while it was found abundant in the two PT-cell culture media. The treatment of single-component solutions of different organic molecules confirmed that L-tyrosine is involved in H_2O_2 release after oxidation by other ROS (presumably O_3 or $^{\bullet}O$), plasma-generated in absence of humidity. The results reported show the extreme importance of properly adjusting the plasma operating conditions depending on the specific chemical composition of the liquid to be treated. Oxidation of biomolecules that are normally consumed by cells may also be another tool for dosing PTWS-induced oxidative stress to cells, in parallel to the administration of free RONS in the medium.[59,64] We believe that our results are of high importance for understanding the dynamic evolution of the chemical composition of PTWS or in general of plasma treatments utilized in the biomedical field to deliver RONS to cells and tissues, and for implementing their potential use in clinics.

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CONFLICT OF INTERESTS

The authors declare that there are no conflicts of interests.

AUTHOR CONTRIBUTIONS

Valeria Veronico was involved in conceptualization, investigation, writing of the original draft and review editing. Eloisa Sardella was involved in supervising the work, conceptualization and data curing. Francesco Fracassi, Pietro Favia and Eloisa Sardella were involved in funds acquisition. Eloisa Sardella, Pietro Favia and Roberto Gristina were involved in the review editing.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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