Effects of Atmosphere Composition and Liquid Type on Plasma-Generated Reactive Species in Biologically Relevant Solutions

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ABSTRACT: This study investigates the reactive species generation by an atmospheric pressure argon plasma jet in biologically relevant liquids. The plasma jet is shielded from the atmosphere by different mixtures of oxygen and nitrogen gas. Different liquids with increasing complexity in ingredient composition (namely, sodium chloride solution, Dulbecco's phosphate-buffered saline solution, and Roswell Park Memorial Institute cell culture medium) were plasma treated and the formation of reactive species was studied. By varying the shielding gas composition, the type and quantity of generated reactive species, reactive nitrogen species, or reactive oxygen species can be tailored. This study shows that the ingredients of the plasma-treated liquid strongly influence the reactive species formation.

KEY WORDS: reactive species, radicals, electron paramagnetic resonance spectroscopy, electron spin resonance spectroscopy, pH, hydrogen peroxide, reactive oxygen species, reactive nitrogen species

I. INTRODUCTION

The relevance of plasma-treated liquids in the field of plasma medicine has gained attention in recent years.[1] Physiological solutions and cell culture media are of great significance because most cells are in a liquid environment or contain liquids. Cold atmospheric pressure plasmas produce reactive species such as free radicals ($^\bullet$OH, $O_2^{\bullet-}$) and other reactive species such as hydrogen peroxide (H_2O_2), nitrate, and nitrite in solutions during treatment.[2,3] In many cases of plasma–liquid interaction, an acidification effect of the liquid is described.[4]

During plasma treatment of liquids at ambient conditions, species originating from the surrounding atmosphere can be transported into the liquid. For plasma jets, oxygen- and nitrogen-containing species can be transported by diffusion and convection into the plasma jet effluent region and can react there to reactive oxygen species (ROS) and/ or reactive nitrogen species (RNS) that subsequently interact with the liquid. Previous studies showed that controlling the ambient composition affects the cellular response to plasma treatment. For example, the viability of human keratinocytes can be modified by a designed plasma atmosphere composition.[5,6] This study takes a closer look at plasma-treated liquids under a controlled gas atmosphere. This research focuses on ROS and

RNS due to their vital role in mammalian systems and because nitrogen and oxygen are the main constituents of ambient air. We investigated an argon plasma jet with a controlled surrounding gas curtain in the treatment of different cell relevant liquids. The surrounding gas curtain composition of oxygen and nitrogen varied from pure nitrogen to pure oxygen as well as different ratios of the two.

II. MATERIALS AND METHODS

A. Plasma Jet and Shielding

The kINPen atmospheric pressure argon plasma jet (neoplas GmbH, Greifswald, Germany) was used in this study. The kINPen consists of a concentric powered rod electrode surrounded by a ceramic capillary. A grounded ring electrode is positioned at a distance of 2 mm before the end of the capillary.[7] The rod electrode is connected to high-frequency power (approximately 1 MHz and 2–6 kV_{pp}). The applied feed gas was high-purity argon (argon N50; Air Liquide, Paris, France) with a gas flow rate of 3 standard liters per minute (slm). A shielding gas device, described by Reuter *et al.*,[8] was utilized to adjust a known atmosphere around the plasma effluent. For this device, a varying shielding gas mixture of oxygen (oxygen N48; Air Liquide) and nitrogen (nitrogen N50; Air Liquide, Paris, France) with a total gas flow rate of 5 slm was applied.

B. Nitrate and Nitrite Assay

A well-known method utilizing Griess reagents was used to determine plasma-generated nitrate (NO_3^-) and nitrite (NO_2^-).[9] A commercial colorimetric assay (nitrite/nitrate detection kit; Cayman Chemical Co., Ann Arbor, MI) was used for the measurements. Here, Griess reagents 1 and 2 react with nitrite to form a purple azo compound. To detect nitrate, this needs to be converted by a reductase enzyme and cofactor to the detectable nitrite. The assay is a simple method to detect the total nitrate/nitrite concentration, which also correlates with the nitric oxide ($\cdot$NO) content in the liquid and in itself is a very important biologically active molecule.

The measurements were performed in four 96-well plates, two for each species. Each data point consists of three independent samples; each sample was performed in triplicate so that each data point is composed of nine analyzed samples. A specific standard curve was also measured for each data point by mixing a defined standard concentration into the liquid.

The standard curve was diluted eightfold to 109.375 µM from original maximal standard concentration of 875 µM. The determination of the concentration was performed by measuring the light absorbance at 540 nm, using a microplate reader (Tecan Infinite M200 Pro; Tecan Group Ltd., Männedorf, Switzerland). The absorbance of the samples compared to the related standard curve yields the respective species concentration.

As described above, nitrate must be converted into nitrite in order to be detectable. Hence, nitrate will be reduced to nitrite by a nitrate reductase enzyme and a related co-

factor. Subsequently, the difference between the total concentration of nitrate and nitrite and the concentration of nitrite yields the amount of nitrate in the plasma-treated liquid.

To compensate for the admixed liquid volume required for the reduction of nitrate, the same amount of untreated solution (20 μL) was admixed to the plasma-treated solution (80 μL) for the nitrite measurement. For the reduction, 10 μL nitrate reductase enzyme and 10 μL nitrate cofactor were admixed. Activation of the samples and corresponding standard curve measurements were performed immediately after the plasma treatment. Griess reagents 1 and 2 were added after 1 h of incubation time; after an additional 10 min, the concentration was determined by measuring the light absorbance. The background signals from the untreated solution and well plate were subtracted.

C. pH Measurement

A pH meter (SevenMulti M47; Mettler-Toledo International Inc., Columbus, OH) was used to measure pH values. Determination of the pH was performed in parallel to the nitrate/nitrite colorimetric assay. Similar to all analytical methods used, each value represents three independent samples treated under the same conditions with the same parameters.

D. H_2O_2 Measurement

H_2O_2 was measured via commercial H_2O_2 test strips (Merckoquant 110011; Merck, Darmstadt, Germany). The test strips were read out by a camera setup in a blackened box to exclude changes in the illumination from incident light. Detailed information about the method and calibration is described by Winter *et al.*[6] On each test stripe, a 20 μL drop of the sample was placed and incubated for 1 min. It has previously shown[6] that the test strips yield the same results as colorimetric assays such as Amplex Red or titanylsulfate measurements for the H_2O_2 concentrations achieved in this case.

E. Electron Paramagnetic Resonance Spectroscopy

A specific detection of free radicals in liquids is possible using electron paramagnetic resonance spectroscopy (EPR). This study used an X-band EPR device (EMXmicro; Bruker BioSpin GmbH, Rheinstetten, Germany). A more detailed description of the procedure is available in a previously published study.[10]

Free radicals are complex to observe due to their short lifetimes. Hydroxyl radicals (˙OH) in solution for example have a lifetime of only a few nanoseconds.[11] A well-known technique, the spin trapping method,[12] was applied. The spin trap, a chemical agent, reacts with free radicals in liquids and forms an adduct, which has a longer lifetime and can thus be measured via electron spin resonance spectroscopy. In this study, the commonly used spin trap DMPO (5,5-dimethy-1-pyrroline-*N*-oxide) from Dojindo Laboratoire (Kumamoto, Japan) was used. DMPO was solved in phosphate-buffered solution (100 mM) and treated with the kINPen for 3 min for different shielding gas variations. A signal of the untreated spin trap solution was observed and subtracted as

a background from the signal of the treated sample. To evaluate the plasma-generated radical concentration, simulated spectra were analyzed. The species concentrations were calculated from the simulated spectra.

III. RESULTS AND DISCUSSION

Fig. 1 depicts the nitrite (NO_2^-) concentration for sodium chloride (NaCl, Sigma Aldrich, St. Louis, MO, USA) solution, Dulbecco's phosphate-buffered saline (DPBS, Lonza, Switzerland); (RPMI, Lonza, Switzerland) solution, and Roswell Park Memorial Institute (RPMI) cell culture medium after 3 min of plasma treatment for different shielding gas ratios of oxygen and nitrogen. RPMI medium and NaCl solution show the highest concentration of NO_2^- for 25% oxygen and 75% nitrogen in the shielding device, whereas DPBS has its maximum of nitrite for the 1:1 ratio of O_2 and N_2 in the shielding gas. A comparison of the amount of generated nitrite in the solution showed that in RPMI, the concentration of NO_2^- is higher for each shielding gas condition than for the other two investigated liquids. The NO_2^- concentration in DPBS is lower than in NaCl solution. In addition, the maximum as a function of shielding gas composition is slightly shifted to a 1:1 ratio of oxygen to nitrogen compared with RPMI medium and NaCl solution, in which the maximum is situated at 25% oxygen to 75% nitrogen in the shielding gas. Fig. 2 shows the nitrate concentration for only sodium chloride solution and DPBS after 3 min of plasma treatment. No nitrate values are shown for RPMI because the small changes in nitrate induced by the plasma cannot be detected with such a high background nitrate concentration in the untreated cell culture medium itself. A comparison of the absolute amount of generated nitrate with the amount of nitrite (Fig. 1) in NaCl and in DPBS, respectively, shows that the order of magnitude is the same for both RNS. Therefore, it must be presumed that both nitrate and nitrite are generated in approximately the same amount. The trend for both liquids over the variation of the shielding gas composition is similar; in addition, the behavior of NO_2^- and NO_3^- in NaCl is alike. For the case of pure nitrogen in the shielding device, the concentration of plasma generated nitrite respectively nitrate, in all liquids, is below the detection limit (2.5 µM) of this assay. For pure oxygen in the shielding gas, the amount of NO_2^- and NO_3^- is the lowest. For example, DPBS shows no nitrate concentration within the detection limit and only a small amount of nitrite could be detected. This behavior could be explained by the fact that a large amount of ozone (O_3) is generated by plasma treatment in the gas phase for 100% O_2 in the shielding gas (Schmidt-Bleker, personal communication). O_3 is known to destroy nitric oxide ($^{\bullet}NO$) and thus to inhibit the formation of nitrite and nitrate, which are final products of $^{\bullet}NO$. The fact that RNS are detectable although no nitrogen was admixed in the shielding leads to the assumption that there are additional sources of nitrogen. One source is the nitrogen from the air dissolved in the liquid; another source could be the impurities of the gases used. However, the feed gas argon and the shielding gas oxygen contain <5 parts per million by volume (ppmv) of nitrogen. In addition to these sources, the RPMI cell culture medium also contains ingredients that contain nitrogen (Table 1). The reason for the different concentrations in the liquids is shown in the strongly differing compositions of the solutions (Table 1).
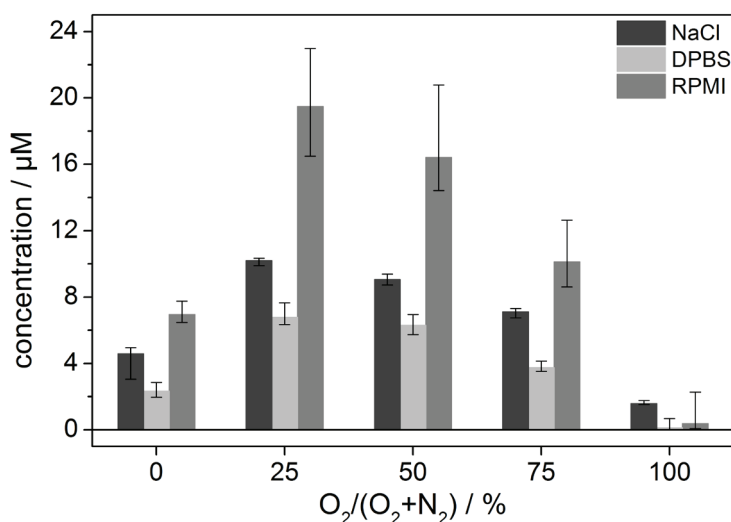
FIG. 1: NO_2^- concentration after 3-min plasma treatment with different ratios of shielding gas composition of DPBS, RPMI, and NaCl solutions.
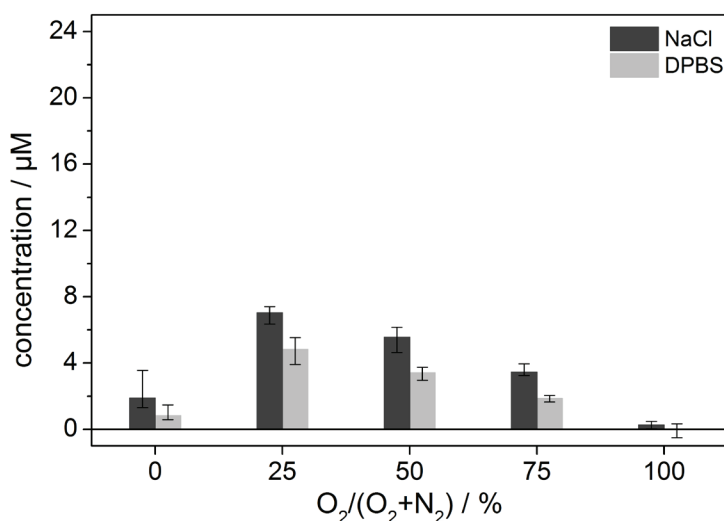

FIG. 2: NO_3^- concentration after 3-min plasma treatment with different ratios of shielding gas composition of DPBS, RPMI, and NaCl solutions.

DPBS contains additional ingredients besides NaCl, such as the two phosphate buffer sodium phosphate dibasic (Na_2HPO_4) and monopotassium phosphate (KH_2PO_4), which are further sources of ROS. The RPMI cell culture medium contains a phosphate buffer (Na_2HPO_4), as well as amino acids and vitamins, which are known as radical scavengers.[13,14] Additional ingredients include calcium nitrate [$Ca(NO_3)_2$], which has an initial concentration of 0.75 mM in the cell culture medium. This concentration is

TABLE 1: Ingredients of NaCl, DPBS, and RPMI solutions

NaCl	DPBS	RPMI
NaCl	NaCl	NaCl
	KCl	KCl
	Na_2HPO_4	$Na_2PO_4 \cdot 7H_2O$
	KH_2PO_4	$Ca(NO_3)_2 \cdot 4H_2O$
		$MgSO_4 \cdot 7H_2O$
		$NaHCO_3$
		Glucose
		Glutathione
		Phenol red·Na
		Amino acids
		Vitamins

more than three orders of magnitude higher than the quantity of nitrate formed by the plasma treatment. Therefore, the small changes in nitrate induced by the plasma cannot be detected with such a high background nitrate concentration in RPMI. However, following Eq. 1, the calcium nitrate in RPMI is an additional source of nitrite after plasma treatment, especially due to its high concentration.

$$NO_2^- + H_2O \leftrightarrow NO_3^- + 2\,H^+ + 2\,e^-$$ (1)
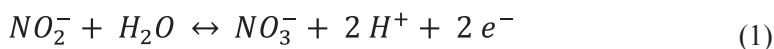
This causes the higher amount of detected nitrite compared to the other two liquids.

The reason that less nitrite and less nitrate are formed in DPBS after plasma treatment can be attributed to a more oxygen-dominated formation of reactive species. This is supported by EPR measurements. Fig. 3 shows the EPR spectra of NaCl solution, DPBS, and RPMI after 3 min of treatment with 100% oxygen as the shielding gas. A simulated spectrum is also depicted for DPBS. The four peaks, which are visible in all three spectra, have an intensity ratio of 1:2:2:1. This peak pattern is characteristic for the hydroxyl radical adduct of the DMPO spin trap. In Fig. 3, these peaks are marked with gray dashed lines. Further peaks are present in the spectra of DPBS, which are attributed to the superoxide anion radical adduct of DMPO (Fig. 3, dotted line).

Fig. 4 shows the acidification of NaCl, DPBS, and RPMI as a function of the oxygen to nitrogen ratio in the shielding gas. The pH value of the DPBS solution is constant over the shielding gas composition variation, due to its buffer capacity. The RPMI cell culture medium reacts differently on the varying surrounding atmosphere, with a slight increase of the pH value compared with the untreated control. This can be attributed to the behavior of an additional ingredient of RPMI, the carbonate buffer sodium bicarbonate ($NaHCO_3$). The pH increase is due to a degassing of the carbonate buffer by the high argon and shielding gas flow of the plasma jet.[15] The increase of the pH value can also be attributed to the loss of the carbonate buffer by reaction with the plasma-generated hydroxyl radicals (Eqs. 2–5).
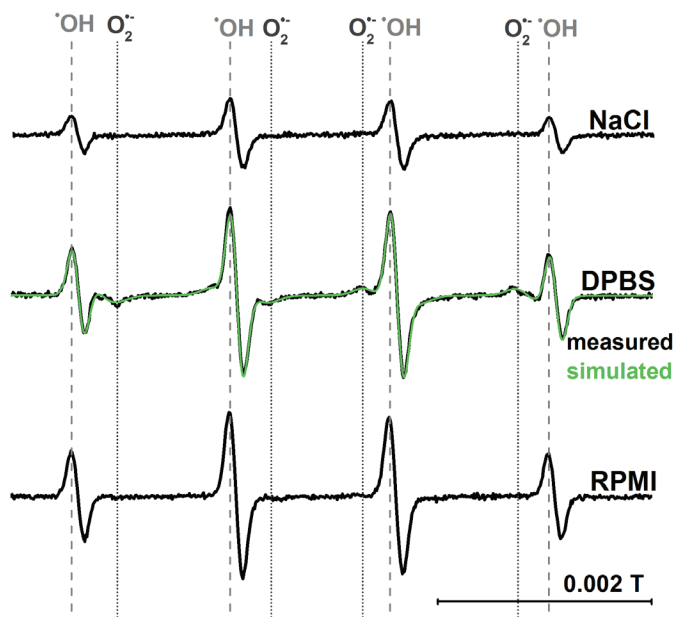
FIG. 3: OH and $O_2^{\cdot-}$ EPR signal after 3-min plasma treatment (100% O_2 as shielding gas composition) of different liquids. The determined total radical concentration [$^{\cdot}OH + O_2^{\cdot-}$] is shown in Table 2.
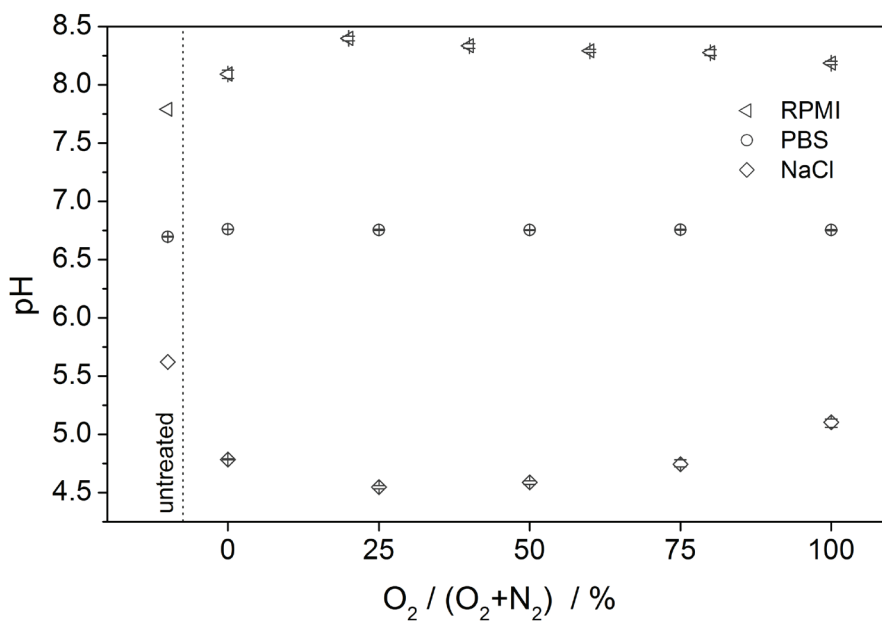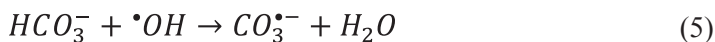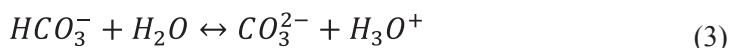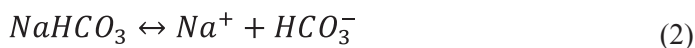

FIG. 4: pH values after 3-min plasma treatment with shielding gas (different O_2/N_2 compositions) for NaCl, RMPI, and DPBS solutions.

$$NaHCO_3 \leftrightarrow Na^+ + HCO_3^-$$ (2)

$$HCO_3^- + H_2O \leftrightarrow CO_3^{2-} + H_3O^+$$ (3)

$$CO_3^{2-} + {}^\bullet OH \rightarrow CO_3^{\bullet -} + OH^-$$ (4)

$$HCO_3^- + {}^\bullet OH \rightarrow CO_3^{\bullet -} + H_2O$$ (5)

The bicarbonate buffer formed by the sodium ion (Na^+) and the bicarbonate ion (HCO_3^-) then reacts with hydroxyl radicals to form carbonate radicals ($CO_3^{\bullet -}$) and the hydronium ion (H_3O^+). The bicarbonate ion can also react with water to form carbonate (CO_3^{2-}) and hydronium ion. This reaction plays only a minor role because the pH is so high that the left side dominates the equation; thus, almost no carbonate should be present in the solutions, which leads to a minor role of Eq. 4.

NaCl solution becomes more acidic after plasma treatment, which was previously described in the literature.[4] Because the pH value never drops below 4.5, almost no HNO_2 is generated in the plasma-treated solutions and only its salt (NO_2^-) is present. In the presence of H_2O_2, nitrite will react into nitrate for acidic conditions (Eq. 6).

$$NO_2^- + H_2O_2 + H^+ \rightarrow ONOOH + H_2O \rightarrow NO_3^- + H_2O$$ (6)
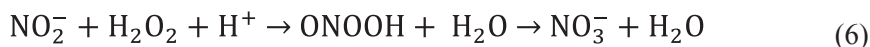
NO_2^- can also decay by reaction with H_2O_2 (Eq. 6) and forms the end product nitrate (NO_3^-) via formation of peroxynitrite ($ONOO^-$) or peroxynitrous acid ($ONOOH$). The role of peroxynitrite for medicine, especially for the inactivation of microorganisms, is described in detail in the literature.[3,16–21] The formation in plasma-treated liquids and the antibacterial effect by the use of plasma-generated peroxynitrite in these liquids is also known.[3,20,22]

Only for the case of plasma treated NaCl solution, the pH value is in a range, where reaction 6 can take place. However, even in this case the value is too high for this reaction to be dominant and a quick decay to nitrate further diminishes the peroxynitrous acid amount.

Fig. 5 shows the H_2O_2 concentration after plasma treatment. For RPMI, DPBS, and NaCl, no dependency of the determined H_2O_2 concentration on the shielding gas composition could be observed. Most H_2O_2 was formed for pure nitrogen as the shielding gas, which seems to be surprising because nitrogen is not involved in the formation of H_2O_2. One possible interpretation is that the higher H_2O_2 concentration generated is not due to this shielding gas composition. Instead, the fundamental change of plasma species concentrations with oxygen in the shielding gas may lead to lower hydrogen peroxide concentrations. Further gas and plasma phase diagnostics need to be performed in future studies. Our finding that more H_2O_2 is formed with nitrogen as the shielding gas leads to the assumption that other less reactive species were generated. H_2O_2 can be formed in water by three reactions, depicted in Eqs. 7–9.[23]
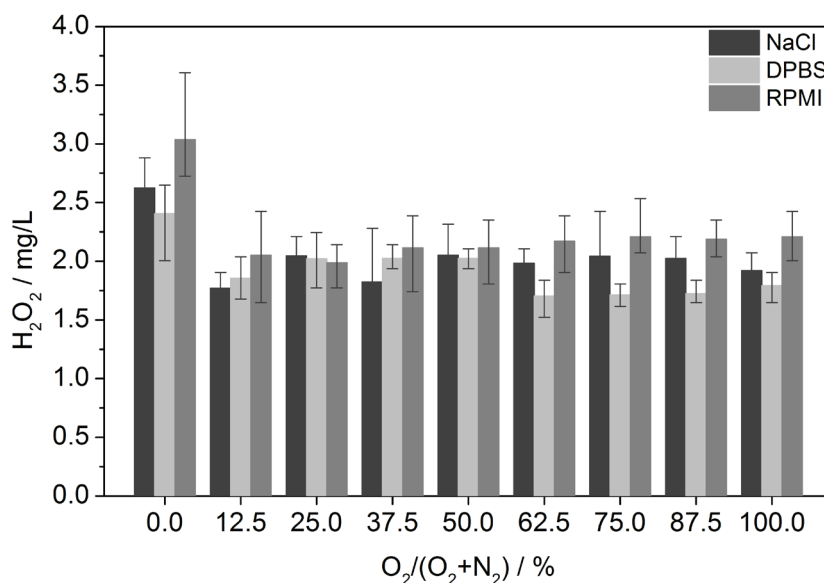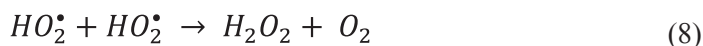
$$^\bullet OH + {}^\bullet OH \rightarrow H_2O_2$$ (7)

FIG. 5: H_2O_2 concentration of different solutions after 3-min plasma treatment with different shielding gas ratios.

$$HO_2^\bullet + HO_2^\bullet \ \rightarrow \ H_2O_2 + \ O_2 \tag{8}$$

$$H^\bullet + HO_2^\bullet \rightarrow H_2O_2 \tag{9}$$

$O_2^{\bullet-}$ and $HO_2^\bullet$ are precursors for H_2O_2 formation. Their pK_a value is 4.8[24]. In the presented experiments, only for NaCl solution treated for 3 minutes at conditions where nitrogen is admixed to the gas curtain, the pH value is lower than 5.0. Therefore formation of hydrogen peroxide by $HO_2^\bullet$ can only occur for NaCl solution treated at these conditions.[24] Therefore, Eqs. 8 and 9 will only occur in sodium chloride solution. In RPMI and DPBS, H_2O_2 is assumed to be mainly generated by hydroxyl radicals.

Table 2 shows a comparison of the radical concentrations in the investigated liquids determined by EPR. The smallest amount of oxygen radicals were formed in sodium chloride solution, followed by RPMI; DPBS had the highest amount of plasma-generated oxygen radicals. Because there is no additional source of oxygen in NaCl, the lowest oxygen radical concentration was expected. In RPMI and DPBS, additional ingredients are further sources for oxygen radicals, which were observed

TABLE 2: Radical concentration after 3-min plasma treatment of different liquids

	NaCl	DPBS	RPMI
[$^\bullet$OH + $O_2^{\bullet-}$], µM	1.9 ± 0.25	5.6 ± 0.5	3.6 ± 0.36

by the EPR measurements. The difference between RPMI and DPBS is because RPMI contains radical scavengers (amino acids and vitamins) that reduce the quantity of plasma-generated oxygen radicals.

IV. CONCLUSION

This study examined the importance of the grade of complexity of plasma-treated liquids as well as the surrounding atmospheric composition for the liquid chemistry. The surrounding atmosphere composition influences the type as well as the quantity of reactive species, which are generated by plasma liquid treatment. A variation of oxygen to nitrogen in the surrounding atmosphere influences both the pH and the nitrate and nitrite concentration, forming a minimum or maximum, respectively, at an oxygen to nitrogen composition of 1 to 3. Furthermore, the surrounding atmosphere as well as the type of liquid define the type and quantity of ROS and RNS generated during plasma treatment. Vital influences have liquid ingredients, which act as a further source of radicals (increasing the total amount of ROS and RNS) or scavenge radicals (reducing the total amount of ROS and RNS). For plasma medicine, it must be taken into account that chemical processes strongly differ if pure saline solution, phosphate buffer, or cell culture medium is used.

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