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Autoreferát dizertačnej práce

**Investigation of cold air plasma generation of aqueous reactive oxygen and nitrogen species with focus on their detection and related antibacterial effects**

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Obhajoba dizertačnej práce sa koná dňa ........................ o ...............h pred komisiou pre obhajobu dizertačnej práce v odbore doktorandského štúdia vymenovanou predsedom odborovej komisie prof. RNDr. Tiborom Hianikom, DrSc. pre študijný odbor 4.1.12 Biofyzika

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# Abstract

In this PhD thesis we investigate formation of reactive oxygen and nitrogen species (RONS) in cold air plasma of transient spark activated water (PAW) and buffered solutions by electrospray and their related antibacterial effect.

DC transient spark discharge was generated in ambient air at atmospheric pressure in combination with water electrospray. The electrospray improved the inter-phase contact and enhanced the transfer of plasma reactive species into the droplets of electrosprayed solutions. The optical emission spectroscopy (OES) of the discharge with electrospray showed a significant number of emission lines: $Fe$, $Fe^+$, $Cr^+$, $Ni^+$, second positive system of $N_2$, $O^+$, $N^+$, $O$ and $N$. We observed the cooling effect due to the water electrospray on the gas temperature of the discharge ($700\ K \rightarrow 400\ K$). Stable gaseous reactive species were identified by Fourier-transform infrared spectroscopy (FTIR), UV absorption and by electrochemical sensors. Transient spark produced mainly nitrogen oxides ($NO + NO_2$) and in the presence of electrospray also other abundant species ($H_2O_2$, $HNO_2$, $HNO_3$, $^{\bullet}OH$, $HO_2^{\bullet}$) were observed. Formation of $O_3$ in other than negligible concentrations was not observed. Formation of aqueous RONS correlated with the production of gaseous reactive species. Detection of aqueous RONS in PAW showed that their formation and life-time depend on the pH. Based on the kinetic chemical analysis we proved formation of peroxynitrous acid, which was determined to be the main antibacterial agent in PAW formed by TS discharge. Highly reactive radicals $^{\bullet}OH$, $HO_2^{\bullet}$, $^{\bullet}NO$ and $^{\bullet}NO_2$ formed in the PAW initiated the decomposition of the cell membranes via the process of lipoperoxidation and resulted in sublethal injury of the cells. We showed that antibacterial properties of PAW can be preserved by immediate deep freeze and storage at low temperatures ($-70°C$). Direct treatment of bacterial suspension in PAW resulted in the strong antibacterial effect (i.e. complete sterilization) and these bacteria showed no metabolic activity due to the synergetic effect of RONS induced chemistry, acidic pH and other plasma agents (e.g. UV emission or electric field).

Formation of peroxynitrite/peroxynitrous acid in PAW was proved also by their fluorescent detection. We investigate the suitability and specificity of 2,7-dischlorodihydrofluorescein diacetate (H$_2$DCFDA) fluorescent dye in plasma activated solutions. We demonstrated that in plasma activated solutions without the presence of $Cl^-$ and thus prohibiting formation of hypochlorite anions ($OCl^-$) or hypochlorous acid (HOCl), the H$_2$DCFDA fluorescence signal can be attributed primarily to $ONOO^-/ONOOH$ formed primary via the reaction of $NO_2^-$ and $H_2O_2$ in PAW. Simple UV-spectroscopic methods for the detection of aqueous $NO_2^-$ and $O_3$ in PAW were examined for their specificity and possible interferences. We confirmed the accuracy of the widely used Griess colorimetric assay for $NO_2^-$ detection and we excluded the interference of $H_2O_2$ on the assay by using the enzyme catalase. By analysis of the phenol degradation products we confirmed that transient spark did not produce aqueous $O_3$ and thus the Indigo blue assay for dissolved $O_3$ detection is non-specific in PAW and strongly interference with the peroxynitrite chemistry in PAW.

# Introduction

Nowadays, non-equilibrium (cold) atmospheric pressure plasmas generated by electrical discharges at the gas-liquid interface or directly in the water have been well studied for different bio-medical, environmental or agricultural applications [1,2]. Water and aqueous solutions (media) treated/activated by cold atmospheric pressure plasmas—so called plasma activated water (PAW) or plasma activated media (PAM)—are currently of great interest for multiple applications in biomedicine and agriculture [3,4], mainly due to their antimicrobial and antitumor properties. Moreover, studying bio-decontamination and other biomedical effects of cold plasmas is very important under wet conditions that are natural to cells. In biomedical plasma applications, such as dentistry, wound healing or tumor treatment, the tissue is usually covered by a thin layer of an aqueous biological liquid. A great number of publications agree that these effects occur thanks to the emerging role of plasma generated reactive oxygen and nitrogen species (RONS), sometimes combined with the effects of other plasma agents (e.g. electric field, electrons and ions, UV radiation), and the RONS induced chemistry [5,6].

Plasmas generated in air or $N_2$–$O_2$ mixtures, or noble gases blown into the ambient air atmosphere, and in contact with liquids generate a number of primary reactive species in the gas phase, e.g. hydroxyl radicals ${}^{\bullet}OH$, hydrogen peroxide $H_2O_2$, nitrogen oxides $N_2O$, $NO$, $NO_2$, ozone $O_3$, and superoxide $O_2^{\bullet-}$. In plasma discharges interacting with water, water evaporation supports the formation of ${}^{\bullet}OH$ and $H_2O_2$ in the gas. These species are transported through the plasma-liquid interface, which induce formation of secondary reactive species in the liquid phase. Secondary RONS such as hydrogen peroxide $H_2O_2$, hydroxyl radical ${}^{\bullet}OH$, nitrites $NO_2^-$, nitrates $NO_3^-$, superoxide/perhydroxyl radical $O_2^{\bullet-}/HO_2^{\bullet}$, peroxynitrite/peroxynitrous acid $ONOO^-$/$ONOOH$ or nitrogen oxides ${}^{\bullet}NO$, $NO_2^{\bullet}$ radicals induce in activated solutions chemical changes and various biocidal effects on microorganisms, or therapeutic effects for biomedical applications [7].

Several studies deal with attempt to identify RONS responsible for the various biomedical effects. However, it is not clear enough yet, which RONS play a dominant role in bio-decontamination or biomedical applications of cold atmospheric plasmas. According some papers, the key species responsible for bactericidal effects may be peroxynitrites/peroxynitrous acid or hydroperoxyl radical [8–10], but other RONS seem to be crucial for medical applications, i.e. nitrogen oxide, superoxide, singlet oxygen, etc. [11–13]. A deeper understanding of the chemical and physical processes occurring when plasma interacts with a liquid surface, particularly with water, represents a key challenge for further studies. In order to control RONS production to obtain the desired effect, it is very important to investigate plasma chemical processes starting in the gas-phase, which affect the chemistry in aqueous phase [14,15]. Better understanding of production of RONS will help us to tune our plasma sources and to choose the best conditions to obtain selective production of RONS with the respect to the type of application and desired expected effect.

# Objectives of the dissertation

The aim of this work is to investigate in detail the effects induced by plasma of electrical discharges in air on bacterial cells in plasma activated water (PAW). The key objective is to understand the atmospheric air plasma chemistry of transient spark with water electrospray and its relation to the properties of plasma activated solutions and subsequent antibacterial effects. This is only possible with proper and precise detection of aqueous RONS in plasma activated solutions.

Production of both gaseous and aqueous reactive species was monitored. The chosen methods for the detection of aqueous RONS were investigated regarding their sensitivity and reliability when using in plasma activated solutions. Bio-decontamination effects induced by transient spark discharge and plasma activated solutions on *E. coli* were investigated. In addition, other examination methods were used in the effort to describe the inactivation process induced by cold air plasma bactericidal agents, which leads to the inactivation/death of the bacterial cells.

# 1 Characterization of the air transient spark discharge with water electrospray

A positive transient spark (TS) generated in ambient air at atmospheric pressure with water electrospray (ES) was used for the treatment of aqueous solutions or planktonic bacteria suspensions of *Escherichia coli*. TS discharge is a relatively novel type of the discharge, which produces very reactive, non-equilibrium (cold) plasma suitable for various environmental and bio-medical applications. TS can be characterized as a self-pulsing, filamentary discharge with transient character despite the applied direct current high voltage. A DC-driven transient spark in positive polarity was generated in point-to-plane configuration in ambient air at atmospheric pressure. The stressed electrode - needle was placed perpendicular above the grounded stainless steel mesh in the point-to-plane geometry. The needle anode was directly joined by the plastic tube to the syringe placed in the syringe pump, which enabled us to inject the aqueous solutions directly through the TS discharge with the constant flow rate 0.5 mL/min. The inter-electrode distance from the tip of the anode to the mesh cathode was kept at 1 cm. The electrospray (ES) of solutions enhanced the mass transfer of gas phase reactive species into the solutions thanks to the increased surface/volume ratio of the micrometric size water droplets. Due to very short duration of the current pulses in order of ~ tens of ns, the plasma generated by the TS has different properties than the plasma generated by an ordinary spark and can be maintained under low energy conditions [16,17].

**Table 1.1** Summary of characteristic parameters of TS discharge with electrospray for typical treatment conditions (1 cm inter-electrode gap, flow rate 0.5 mL/min).

| $U_{max}$ | $U_{mean}$ | $I_{max}$ | $f$ | $E_{mean}$/pulse | $P_{mean}$ | $E_{mean}$/mL |
|---|---|---|---|---|---|---|
| 11-13 kV | 9-11 kV | 25-30 A | 1 kHz | 2 mJ | 2 W | 240 J/mL |

# 2    Optical emission spectroscopy of the transient spark with water electrospray

The optical emission spectroscopy (OES) of the TS discharge without electrospray or TS discharge above the water surface have been already investigated. However, we performed for the first time a time-integrated optical emission spectroscopy of the TS discharge with and without water electrospray in order to discover how the presence of electrospray affects the production of reactive species generated by the TS discharge. Moreover, we determined the rotational $T_r$ and vibrational $T_v$ temperatures from the measured emission spectra.

For fast recording of time-integrated spectra of a broad spectral region we used a two-channel compact emission spectrometer *Ocean Optics SD2000* with CCD detector, which allowed us scanning the spectra through the spectral range from UV to NIR (200-1100 nm with the resolution 0.6-1.7 nm). The comparison of typical emission spectra of TS discharge (1 kHz, 1cm gap, 0.5 mL/min flow rate) in UV-VIS and in VIS-NIR region for position 0 mm (directly below the anode tip) shows, that the presence of water ES through the discharge altered the emission spectra (**Fig. 1**):

- the emission in UV region (mainly <300 nm) in the presence of ES was significantly enhanced and new emission lines were observed;
- the intensity of emission lines in the area 350-500 nm range partially decreased in the presence of ES and few new emission lines were observed;
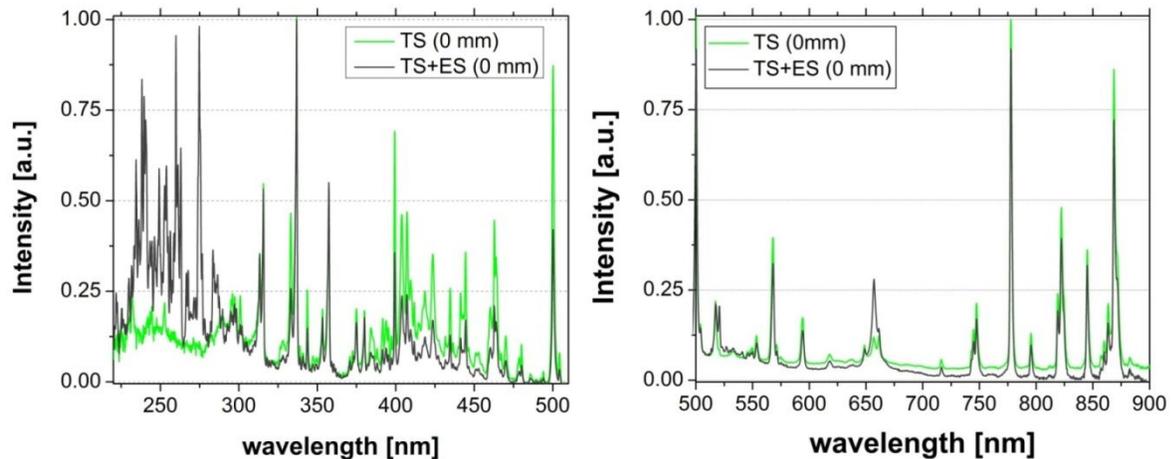- the intensity of the emission lines above 500 nm did not change in the presence of ES.



**Figure 1** UV-VIS (left) and VIS-NIR (right) emission spectra of TS discharge with (TS+ES) and without electrospray (TS) for position directly below the needle electrode (1 cm gap, 0.5 mL/min) (normalized for $N_2$ SPS (0-0) at 337 nm).

Furthermore, we identified the main emission lines in the UV-VIS-NIR spectrum for TS+ES in position 0 mm by using NIST database [18]. The UV spectrum below 300 nm was dominated by emission lines of metal ions $Fe^+$, Fe, but also $Cr^+$ and $Ni^+$. Few lines of metal ions were observed also in VIS area up to 550 nm. The HV electrode - needle, which we typically use, is

made of stainless, chromium-nickel steel with silicone coating. These results confirmed, that the emission below 300 nm is due to the sputtered material of needle anode and this process is also enhanced by the water ES. Beside this, the emission spectra below 600 nm were dominated by second positive system of $N_2$ ($N_2$ SPS) and by emission lines of $O^+$ and $N^+$ ions; NIR spectra (> 700 nm) were dominated by atomic O and N lines. Emission of $NO_\gamma$ (A-X) bands below 300 nm, which are often observed in air plasmas, was not observed probably due to the overlap with the strong emission of metallic ions from the anode in UV. Furthermore, for cold plasmas generated in ambient air with presence of water vapors, OH (A-X) band in UV (306-310) are typically observed due to water dissociation. Despite the presence of water ES, which is responsible for the increase of the relative humidity up to ~70%, we did not observe the OH bands in the emission spectra. One reason could be that OH (A-X) band are overlapped with the $N_2$ SPS 1-0 and 2-1 bands [19]. However, in [20] it was estimated that electronic quenching of OH(A–X) by water reduces the effective lifetime of OH(A) drastically.

The gas temperature of the discharge is important from the point of view of the plasma induced gas chemistry, which typically depends on the temperature. We used the measured time-resolved spectra of the $N_2$ SPS positive system to determine the gas temperature $T_g$ of the discharge, assuming that it approximately equals the rotational temperature $T_r$ of excited $N_2$ (C) species. We showed that the electrospray of water through the discharge decreased both rotational and vibrational temperatures ($T_r$ and $T_v$). Since $T_r$ is close to the gas temperature $T_g$, we can see that the electrospray cooled down $T_g$ from ~700 K to ~400 K ($T_v$ decreased from 3700 K to 3200 K respectively).
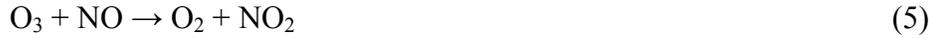
# 3 Gas-liquid induced chemistry of air transient spark with water electrospray and related antibacterial effects

In this chapter we provide a closer look on the transient spark discharge gas phase chemistry and detection of gaseous RONS, and on their transfer into the electrosprayed solution followed by the investigation of the main bactericidal agents of our transient spark and PAW.

## 3.1 Gas-phase chemistry of transient spark with and without electrospray

TS discharge with or without water electrospray was operated under typical conditions (1 cm gap, 1 kHz) in the open reactor in ambient air at atmospheric pressure. Detection of gaseous reactive species produced by cold air plasma of TS discharge was investigated by means of Fourier-transform infrared (FTIR) spectroscopy, UV absorption and electrochemical sensors. We also described the chemistry leading to the production of detected gaseous reactive species.
As the dominant stable gas phase products of TS discharge were identified nitrogen oxides (NO and $NO_2$), while ozone was negligible (<10 ppm detection limit). During the rising slope of the current pulses (both streamer and spark), electrons have enough energy (temperature) to ionize

and dissociate dominant air molecules: $N_2$ and $O_2$. Atomic oxygen $^\bullet O$ is the major intermediate product, because the direct electron impact dissociation of $N_2$ is much slower than of $O_2$. Fast quenching of the excited $N_2^*$ molecules with molecular oxygen is the additional sources of atomic $^\bullet O$. Formation of NO can proceed via Zeldovich mechanism (*1*) and (*2*). The production of atomic oxygen species O resulted into the further oxidation of NO into $NO_2$ in a three-body reaction (*3*). Furthermore, at low gas temperature accumulated O atoms may also generate ozone $O_3$ via slow 3-body reaction (*4*), which is further able to oxidize NO to $NO_2$ (*5*).

$$^\bullet N + O_2 \rightarrow NO + {}^\bullet O \tag{1}$$
$$^\bullet O + N_2 \rightarrow NO + {}^\bullet N \tag{2}$$
$$NO + {}^\bullet O + M \rightarrow NO_2 + M \tag{3}$$
$$^\bullet O + O_2 + M \rightarrow O_3 + M \tag{4}$$
$$O_3 + NO \rightarrow O_2 + NO_2 \tag{5}$$

Applying the water electrospray through the discharge affected the gas-phase chemistry. In TS discharge with water ES, $H_2O$ molecules were dissociated by electron impact and produced $^\bullet OH$ or $HO_2^\bullet$ radicals, resulting in gaseous $H_2O_2$ that interacted with NO and $NO_2$ and influenced their measured gas-phase concentrations and lead to formation of gaseous $HNO_2$ and $HNO_3$. In higher power transient spark, $O_3$ formation was suppressed and the gas products are dominated by nitrogen oxides (NO and $NO_2$).

**Figure 2** shows the concentrations of gaseous NO and $NO_2$ generated by TS discharge in the ambient air without (TS) and with water ES (TS+ES). Concentrations of NO were in general much higher than $NO_2$ due to fact that NO is the primary product of Zeldovich mechanism, while $NO_2$ is formed by later NO oxidation with $^\bullet O$ or $O_3$, which is much slower and cannot oxidize most of the formed NO, as there is not a sufficient time between the TS discharge pulses [21]. The NO concentration generated by TS was found considerably lower in air humidified by the water electrospray. The $NO_x$ dissolution (driven by the Henry's law equilibria) into the electrosprayed water microdroplets depleted $NO_x$ from the air. The water ES through the plasma improved the gas-liquid transport of the gaseous $NO_x$ into the liquid resulting in $NO_x$ dissolution in the water.
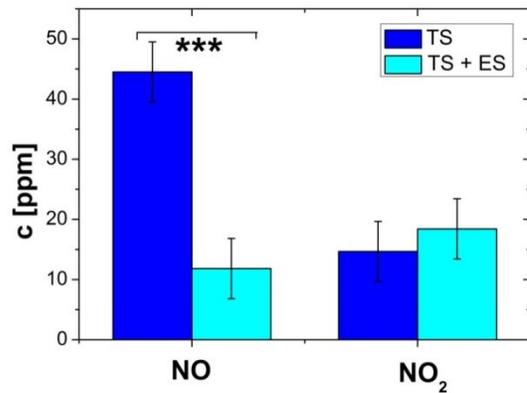


**Figure 2** Nitrogen oxides (NO and $NO_2$) concentrations produced by TS discharge at ambient air with (TS+ES) and without (TS) water electrospray (mean +/- SEM, n(TS) = 6, n(TS+ES) = 17); ***significant difference P < 0.0001 (Mann-Whitney test).

## 3.2 Air transient spark induced chemistry in plasma treated aqueous solutions

Formation of aqueous RONS in plasma activated solutions is due to production of reactive species in the gas phase of discharge. Gaseous $H_2O_2$, $HNO_2$ and $HNO_3$, but also NO and $NO_2$ are readily dissolved in water due to their high solubilities, resulting in formation of aqueous RONS in electrosprayed solutions. The concentrations of long-lived species ($H_2O_2$, $NO_2^-$ and $NO_3^-$) were measured in PAW and PAPB. Indirect methods for detection of transient species ($O_2^{\bullet-}$/ $HO_2^{\bullet}$, $ONOO^-$/$ONOOH$) were used. We focused on RONS detection directly after plasma treatment, but we also observed the evolution of their concentrations in post-plasma treatment time. Chemical processes in plasma activated solutions were described and linked with pH changes.

DC transient spark discharge generated in ambient air was in the direct contact with the aqueous phosphate solutions – W (0.85 mM $NaH_2PO_4$) and PB (2 mM $Na_2HPO_4$/$KH_2PO_4$), which were electrosprayed directly through the active zone of discharge and called as plasma activated water (PAW) and plasma activated phosphate buffer (PAPB). Changes of pH, conductivity and formation of RONS were observed in water or PB electrosprayed through TS discharge.
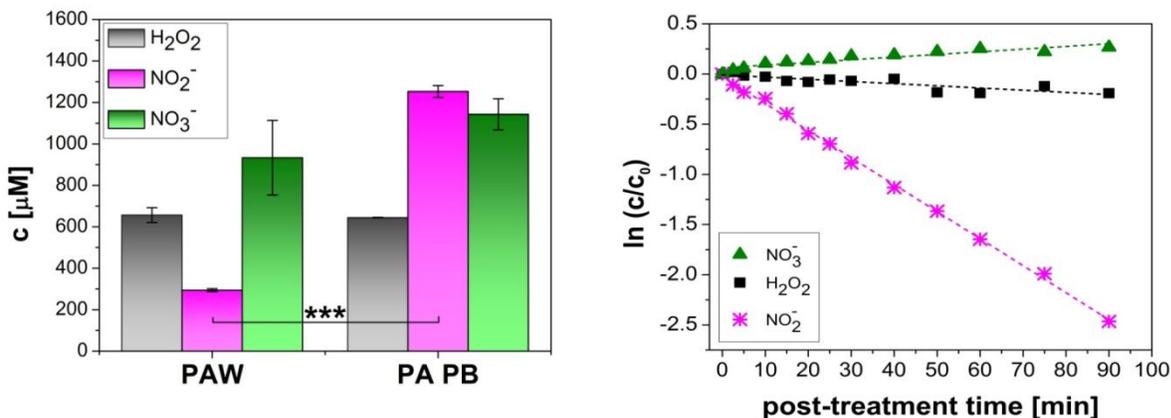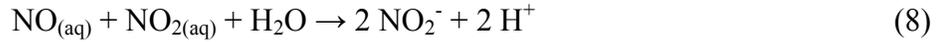


**Figure 3** Left: $H_2O_2$, $NO_2^-$ and $NO_3^-$ concentrations in PAW and PAPB after electrospraying W and PB solutions through transient spark; right: First-order kinetic of post-plasma treatment evolution of $H_2O_2$, $NO_2^-$ and $NO_3^-$ concentrations in PAW up to 90 minutes at room temperature.
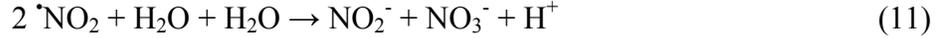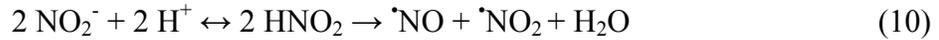
**Figure 3** (left) shows concentrations of $H_2O_2$, $NO_3^-$ and $NO_2^-$ measured in PAW and PAPB solutions directly after typical TS electrospray treatment. In PAW we observed the decrease of pH (5 → 3.2) unlike in plasma activated buffered solution (PAPB), where pH remained at 6.9. Aqueous $H_2O_{2(aq)}$ is produced by extremely fast dissolution of gaseous $H_2O_{2(g)}$ formed in the gas by recombination of $^{\bullet}OH$ radicals (6). $H_2O_2$ has the highest Henry's law solubility coefficient ($k_H \approx 9 \times 10^2$ molm$^{-3}$Pa$^{-1}$), thus all gaseous $H_2O_2$ was readily transferred into solutions through the gas–liquid interface [22,23]. Nitrites $NO_2^-$ and nitrates $NO_3^-$ are generated in the PAW by the dissolution of gaseous NO and $NO_2$ via the following reactions (7) and (8) [9,24]. $NO_2^-$ and $NO_3^-$ can be formed in PAW/PAPB by solvation of gaseous $HNO_2$ and $HNO_3$ due to their high solutbility coefficient (kH $\approx 4.8 \times 10^{-1}$ mol m$^{-3}$ Pa$^{-1}$for $HNO_2$ and extremely high $\approx 2.1 \times 10^3$ mol m$^{-3}$

Pa$^{-1}$ for HNO$_3$). These processes, i.e. formation of NO$_2^-$ and NO$_3^-$ are linked with release of hydrogen protons H$^+$ and therefore they were responsible for the acidification of the plasma activated solutions.

$$^\bullet OH + {}^\bullet OH + M \rightarrow H_2O_2 + M \tag{6}$$

$$NO_{2(aq)} + NO_{2(aq)} + H_2O \rightarrow NO_2^- + NO_3^- + 2\ H^+ \tag{7}$$

$$NO_{(aq)} + NO_{2(aq)} + H_2O \rightarrow 2\ NO_2^- + 2\ H^+ \tag{8}$$

The following processes, which are strongly dependent on pH of solution, were mainly responsible for the different concentrations of NO$_2^-$ in PAW and PAPB, and also for decay of long-lived species NO$_2^-$ and H$_2$O$_2$ in PAW in post-plasma treatment time (**Figure 3** (right)):

1. reaction of H$_2$O$_2$ in acidic environment with NO$_2^-$ led to the formation of the peroxynitrites/peroxynitrous acid (ONOO$^-$/ONOOH) (*9*). Peroxynitrite anion and peroxynitrous acid exist in equilibrium and their presence in solution depend strongly on the pH of the solution (p$K_a$ (ONOO$^-$/ONOOH) = 6.8). In our PAW we expect due to pH = 3.2 more than 99% of peroxynitrites to be presented in the form of acid.

2. NO$_2^-$ ("acidified nitrites") are not stable and decompose under acidic conditions (pH < 3.5) via formation of $^\bullet$NO and $^\bullet$NO$_2$ intermediates (*10*) into NO$_3^-$ anions as final products (*11*).

$$NO_2^- + H_2O_2 + H^+ \rightarrow O{=}NOOH + H_2O \tag{9}$$

$$2\ NO_2^- + 2\ H^+ \leftrightarrow 2\ HNO_2 \rightarrow {}^\bullet NO + {}^\bullet NO_2 + H_2O \tag{10}$$

$$2\ {}^\bullet NO_2 + H_2O + H_2O \rightarrow NO_2^- + NO_3^- + H^+ \tag{11}$$

To confirm the formation of peroxynitrites/peroxynitrous acid (ONOO$^-$/ONOOH) we performed the kinetic analysis of post-plasma treatment chemical processes in PAW. If the reaction between H$_2$O$_2$ and NO$_2^-$ represents the main pathway of formation of ONOOH, then according to Lukes *et al.* [24] the rate of peroxynitrite formation $r_{ONOOH}$ can be written as (*12*), where $k$ is the third-order rate constant for reaction (*9*) (assuming that the reaction is first-order with respect to [H$^+$],[HNO$_2$] and [H$_2$O$_2$]).

$$r_{ONOOH} = \frac{d[ONOOH]}{dt} = k[H^+][H_2O_2][NO_2^-] \tag{12}$$

$$ln\frac{[NO_2^-]_t[H_2O_2]_0}{[H_2O_2]_t[NO_2^-]_0} = k_{H_2O_2,NO_2^-}([NO_2^-]_0 - [H_2O_2]_0)t \tag{13}$$
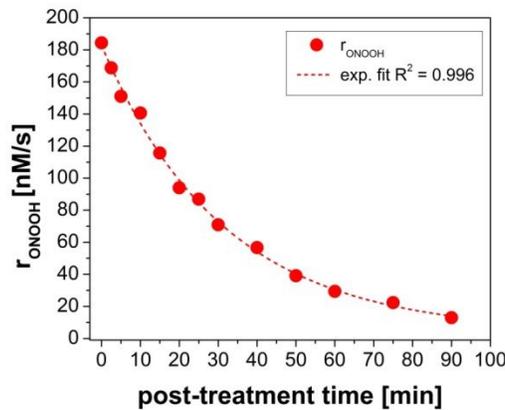


**Figure 4** Kinetic of the rate formation of peroxynitrous acid $r_{ONOOH}$ in PAW (pH 3.2) post plasma treatment.

For given experimental conditions (pH 3.2, room temperature) data showed an excellent linear fit with the second-order reaction between $H_2O_2$ and $NO_2^-$ in PAW. Form the slope of the plot we determined the pseudo-second-order rate constant to be $k_{H_2O_2,NO_2^-} = 1.23$ $M^{-1}s^{-1}$ according equation (13). The value of the third-order rate constant $k$ for reaction (9) was then estimated using the calculated concentration of hydrogen ions $[H^+] = 6.31 \times 10^{-4}$ M for pH 3.2 as $k_{ONOOH} = 1.94 \times 10^3$ $M^{-2}s^{-1}$. From the experimental data of measured concentrations of $H_2O_2$, $NO_2^-$ we determined the rate of formation of peroxynitrous acid $r_{ONOOH}$ in PAW (pH 3.2) for each time point from 0 to 90 min post plasma treatment according the equation (9). The initial rate of ONOOH formation in fresh PAW was determined to be $r_{ONOOH} = 184$ nMs$^{-1}$. As can be seen from **Figure 4**, the rate decreased exponentially in the post-plasma treatment time and after approximately 90 minutes post plasma treatment was the value $r_{ONOOH}$ one order of magnitude lower.

## 3.3 Bactericidal effects induced by air transient spark with electrospray and/or PAW linked with RONS chemistry

We showed that PAW activated by air TS discharge with water electrospray contains both long-lived and transient RONS. Mainly the reactive species like ONOOH, $^\bullet$OH, $^\bullet$NO and $^\bullet$NO$_2$ radicals are known to posses bactericidal properties. We investigated and compared the effects of the direct cold air plasma treatment and/or indirect treatment by plasma activated solutions (mainly PAW) on model Gram-negative bacteria *Eschericchia coli* ATCC 25922. We performed two types of treatments:

- direct treatment when the bacterial suspensions in W or PB solutions were electrosprayed directly through the TS discharge;
- indirect treatment when only aqueous solutions (W and PB) were treated by TS with electrospray. These freshly activated solutions (PAW, PAPB) were then used for treatment of bacteria and the antibactericidal effect in post- plasma treatment time was evaluated.

Beside that we focused also on other effects induced by plasma and PAW like sublethally injured cells with damage of outer and cytoplasmic membrane, the peroxidation of membrane lipids and the metabolic activity. The aim was also to distinguish the plasma agents responsible for the stronger inactivation effect in the case of direct plasma treatment.

Bactericidal effects obtained by the direct treatment of *E. coli* bacterial suspensions in W or PB by TS with ES and by indirect treatment when bacteria were incubated in fresh activated solutions (PAW or PAPB) are shown in **Figure 5** (left). The bactericidal efficiency of TS discharge or PAW/PAPB treatment was evaluated by classic thermostatic cultivation of Petri dishes and determined as a logarithmic reduction of the bacterial population according the equation (14),

$$\log reduction = \log_{10}\left(\frac{C_0}{C}\right) \tag{14}$$

where $C_0$ represented the initial population of bacteria (i.e. non-treated) and $C$ was concentration of bacteria after certain plasma treatment. A significantly higher bactericidal effect

(up to 6-7 logs, i.e. complete sterilization) was obtained by direct treatment of bacteria in W in comparison with bacteria in buffered PB, where we obtained less than 2 logs bactericidal effect. Similarly, strong bactericidal effect was observed after indirect treatment when bacteria were incubated in fresh PAW where the inactivation efficiency was increasing with the increasing incubation time (increase of inactivation from 1.5 logs after 10 min up to 5 logs after 60 min). The indirect treatment by fresh PAPB (pH ~ 6.6-6.8) resulted in very weak bactericidal effect (max. 0.5 log). Formation of aqueous RONS followed by chemical changes are considered as the main bactericidal plasma agent. Clearly the higher bactericidal efficiency was observed in the case, that bacteria were suspended in non-buffered W or treated by PAW.
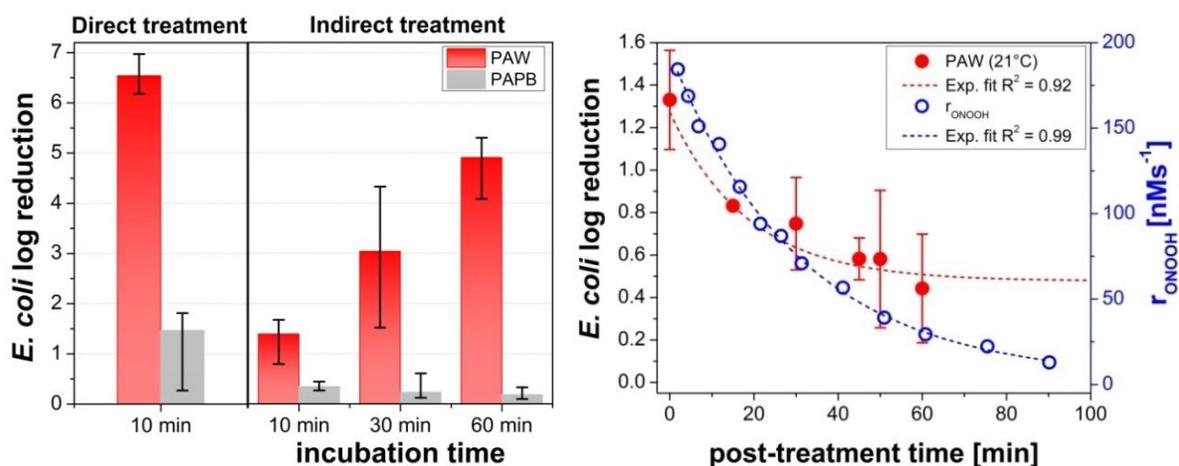


**Figure 5** Left: Comparison of bactericidal effects of direct treatment (TS with ES) and indirect treatment by plasma activated solutions (mean, $1^{st}$ and $3^{rd}$ quartile, n = 6-10); right: Correlation of the exponential decay of bactericidal effect of PAW and the rate constant of ONOOH formation ($r_{ONOOH}$) in PAW in post-plasma treatment time at room temperature.

Formation of aqueous RONS was linked with the acidification of non-buffered W solution (final pH 3.2 for PAW) and different RONS chemistry associated with acidification. This is important especially for the indirect treatment by PAW, where the bactericidal effect is given only by the action of aqueous RONS. Following chemical processes enhanced by the low pH were responsible for the strong bactericidal effects in PAW due to production of highly reactive RONS (i.e. strong oxidants with cytotoxic effects):

1. Production of peroxynitrous acid ONOOH: Life-time and the reactivity of $ONOOH/ONOO^-$ is strongly pH-dependent. ONOOH decays rapidly by homolysis to $^\bullet OH$ and $^\bullet NO_2$ radicals (*15*). biological systems, $ONOOH/ONOO^-$ promotes the oxidative modification of target molecules either by the direct oxidation reaction (one- or two-electron oxidation) or by the radicals derived from the ONOOH homolysis. This decomposition seems to become relevant in hydrophobic phase of the cell membrane (i.e. when crosses the membrane by passive diffusion), where the decay radicals initiate the lipid peroxidation, and lipid and protein nitration processes [25,26].

$$O=NOOH \leftrightarrow {}^\bullet OH + {}^\bullet NO_2 \qquad (15)$$

2. Formation of $^\bullet NO$ and $^\bullet NO_2$ radicals due to of acidic decomposition (*10, 11*) process of $NO_2^-$ to $NO_3^-$ (i.e. acidified nitrites), which is enhanced at pH < 3.5 [5]. Nitrogen oxides lead to the nitration of the polyunsaturated fatty acids in the cell membranes.

3. Formation of hydroperoxyl radical $HO_2^\bullet$ due to acidified pH of our PAW which can as an uncharged species easily permeate bacterial cells by traversing the hydrophobic cell membrane, unlike the $O_2^{\bullet-}$ radical. $HO_2^\bullet$ can then denature proteins inside the cell by chemical modification, causing bacterial inactivation [8].

Previously we have shown, that the concentration of $NO_2^-$ and $H_2O_2$ were not stable and decayed exponentially in post-treatment time to form the ONOOH acid. In **Figure 5** (right) is shown the correlation of the bactericidal properties of PAW and of the calculated rate constant $r_{ONOOH}$ in post-treatment time up to 90 minutes at the room temperature. Clearly we can see a similarity in the exponential decay of both the bactericidal effect and formation of ONOOH in PAW and it can be assumed, that the peroxynitrite chemistry dominates in PAW produced by air transient spark with electrospray.

Mostly radicals $^\bullet OH$, $^\bullet NO$, $^\bullet NO_2$ and $HO_2^\bullet$, which are typically produced by plasma induced aqueous chemistry in treated solutions, are considered as direct or indirect initiators of the lipid peroxidation resulted in oxidative damage of the cell membrane. Sublethal injury typically appears when cells are subjected to the physical or chemical agents insufficient to kill them. However, the cells become sensitized and less resistant to other bactericidal agents. The sublethal injury is associated with the membrane damage. Model *E. coli* bacteria are Gram-negative bacteria, have except the cytoplasmatic (inner) cell membrane (CM) in addition the outer cell membrane (OM) composed of lipopolysaccharides, proteins and phospholipids. We observed sublethal injury of both cell membranes that was caused only due to the action of chemical agents (i.e. aqueous RONS and acidic pH) in PAW, which are in addition responsible for the oxidative damage of the cell membranes. This means that although the bacteria survived and were able to growth after indirect treatment by PAW, their outer and cytoplasmatic membranes are damaged, and the bacteria are now more sensitive to other environmental factors.

Moreover, in **Figure 5** (left) is clearly remarkable difference in bactericidal efficiency obtained by the direct treatment of bacteria in W (~6-7 logs) compared to the 10 min indirect treatment by PAW (~1.5 log). In the case of direct treatment another active agents of cold plasma of transient spark discharge beside the RONS need to be considered and examined in the future, e.g. UV radiation, electric field and charged particles, metallic (nano)particles.

# 4 Investigation of detection methods of reactive oxygen and nitrogen species in plasma activated aqueous solutions

For every application of plasma activated solution it is important to know their properties and limitations, which can only be determined by the accurate detection of every single RONS. However, the RONS detection may be often difficult due to their very short life-times, strong

reactivity, low concentrations, etc. All pros and cons should be taken in account when selecting the proper method for detection of aqueous RONS in plasma activated solutions. However, for many methods it is not clear, whether they are suitable to be used in such a specific conditions of PAW/PAM.

## 4.1 Fluorescent measurement of peroxynitrites/peroxynitrous acid in cold air plasma treated aqueous solutions

The detection of peroxynitrites in general is difficult because of their high reactivity, very short life-time ranging from milliseconds to seconds, spontaneous decay, and typically very low concentrations (~nM) in biological systems. Likewise, the same problems arise in plasma activated liquids. Despite the present literature highlights the importance of peroxynitrite chemistry for the antimicrobial properties of PAW, up to now only a few papers have demonstrated evidence of the either directly or indirectly measured presence of $ONOO^-$/ONOOH in plasma activated liquids. The present study addresses this possibility of qualitative detection of peroxynitrites/peroxynitrous acid couple ($ONOO^-$/ONOOH) in air atmospheric plasma activated solutions by means of fluorescent spectroscopy using 2,7-dichlorodihydrofluorescein diacetate ($H_2DCFDA$) fluorescent dye. First, possible cross-reactivity of the $H_2DCFDA$ dye with various RONS, which are typically produced in the PAW, were checked. Second, the specificity of various RONS scavengers was investigated with the respect to the $H_2DCFDA$ fluorescent response. Third, the fluorescent response of $H_2DCFDA$ was measured in plasma activated aqueous solutions directly after plasma treatment. To prove that $ONOO^-$/ONOOH are formed in acidified PAW and they are responsible for the strong fluorescent response of the $H_2DCFDA$ dye, RONS scavengers for $H_2O_2$ and $NO_2^-$, the reactant species of the peroxynitrite chemistry, as well as the scavenger of its end-products, were used. Finally, we performed a kinetic study of post-discharge processes in PAW focused on the formation of ONOOH followed by the parallel detection of $ONOO^-$/ONOOH with the $H_2DCFDA$ fluorescent dye.

Many of aqueous RONS are known to be strong oxidants [27], which may lead to false positive fluorescent signals due to oxidant-sensitive character of the dye, if it is not specific enough. As can be seen in **Figure 6** (left), sensitivity of the $H_2DCFDA$ dye to $ONOO^-$/ONOOH is much higher (about 1-2 orders of magnitude) than sensitivity to $OCl^-$/HOCl of the same concentrations. Even the low concentrations of $ONOO^-$/ONOOH resulted in the higher fluorescence response than the same concentrations of $OCl^-$/HOCl. Fluorescent response of other tested RONS ($H_2O_2$, $NO_2^-$, $O_2^{\bullet-}$) was not significant, even though their typical concentrations in plasma activated solutions are in ~hundreds of μM (i.e. $H_2O_2$, $NO_2^-$/ $NO_3^-$) [28].
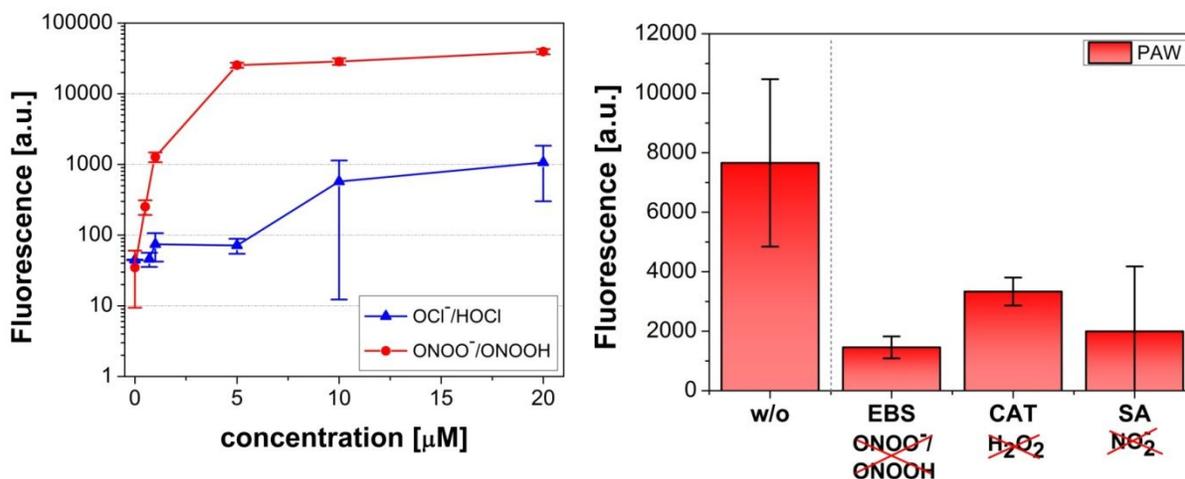
**Figure 6** Left: Fluorescent signal of the H$_2$DCFDA dye to different concentrations of ONOO$^-$/ONOOH and OCl$^-$/HOCl in phosphate buffer at pH 6.9; right: Fluorescent signal of the H$_2$DCFDA dye to different concentrations of ONOO$^-$/ONOOH and OCl$^-$/HOCl in phosphate buffer at pH 6.9.

The efficiencies of scavengers were subsequently tested in the plasma treated solutions with respect to the fluorescence signal with or without scavengers added directly after plasma treatment before mixing with the H$_2$DCFDA dye. By using RONS scavengers we tried to prove that the predominant ONOOH formation was responsible for the strong fluorescent signal of H$_2$DCFDA dye in PAW**.** The used scavengers were aimed to scavenge the reactants or products of ONOO$^-$/ONOOH formation in plasma activated solutions: catalase (CAT) scavenging H$_2$O$_2$, sodium azide (SA) scavenging NO$_2^-$ and ebselen (EBS) scavenging ONOO$^-$/ONOOH. In **Figure 6** (right) we can see that the addition of EBS into the PAW significantly decreased the fluorescence signal. We expected that the missing part of the fluorescent signal in PAW is due to the scavenged ONOOH. By addition of the CAT into the PAW we observed a significant decrease of the H$_2$DCFDA fluorescence intensity. We expect that CAT scavenged H$_2$O$_2$ needed for the formation of ONOOH, and therefore no or minimal concentrations of ONOOH were formed. In the same manner, we used SA as a scavenger of NO$_2^-$. We observed similar decrease of the fluorescent signal due to the scavenging of NO$_2^-$ as one of the reactants needed for ONOOH formation. In both cases, after removal of H$_2$O$_2$ or NO$_2^-$ by scavengers, the decrease of the fluorescent signal is due to absence of ONOOH.  This result supported the assumption that the fluorescent signal of H$_2$DCFDA in PAW is caused by ONOO$^-$/ONOOH formation, which also continues in PAW in post-treatment time.

We performed again the kinetic analysis of post-plasma treatment chemical processes in PAW and the time dependent evolution of ONOOH formation rate $r_{ONOOH}$ in PAW was compared with the time evolution of the measured fluorescent signal of H$_2$DCFDA in PAW in post-discharge time. The trend of H$_2$DCFDA fluorescent signal showed very similar decreasing exponential trend as a result of the decrease of ONOOH concentration due to its acidic decomposition into $^\bullet$OH and $^\bullet$NO$_2$ radicals (*15*) in PAW in post-plasma treatment time. As can be seen in **Figure 7** there is a very good agreement in detection of ONOO$^-$/ONOOH by these two

methods. Hence we calculated the formation rate of ONOOH in ~nM range, the actual concentrations of ONOOH in PAW during the plasma treatment might be higher (ONOOH are continuously formed and decayed). Despite the low expected steady-state concentration of ONOO$^-$/ONOOH in PAW due to their short life-time, the H$_2$DCFDA dye effectively detected these low concentrations, showed its great sensitivity and can serve as a qualitative method for ONOO$^-$/ONOOH detection in different types of PAWs.
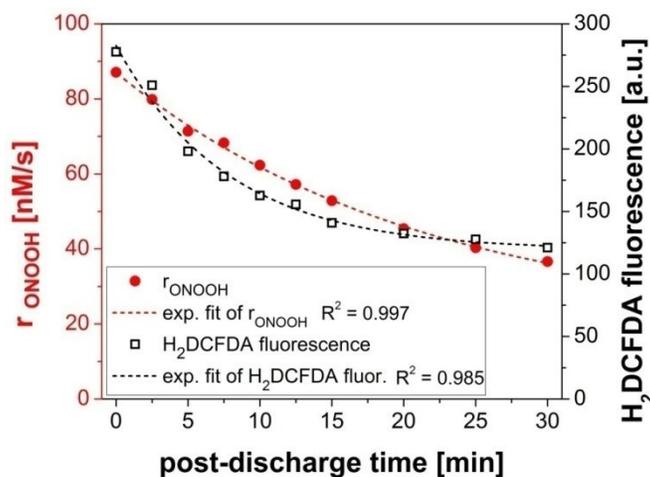


**Figure 7** Parallel detection of ONOO$^-$/ONOOH in PAW (pH 3.2, 10°C) in post-discharge time by the fluorescent dye H$_2$DCFDA and by the kinetic study of ONOOH formation rate $r_{ONOOH}$.

## 4.2    Specificity of detection methods of nitrites and ozone in aqueous solutions activated by air plasma

Nitrites NO$_2^-$ (and nitrates NO$_3^-$) are typically formed in plasma activated solutions by the dissolution of nitrogen oxides from the gas phase and they are linked with the acidification of the solution. Ozone is one of the abundant reactive species formed in air or oxygen plasma discharges with relatively low power, and can dissolve into the liquids. Based on the previous analysis of gaseous products formed by the positive TS discharge generated in ambient air with water electrospray, which showed formation of mainly NO$_x$ and only traces of O$_3$, we focused on the method for nitrites and dissolved ozone detection. We compared the accuracy of the Griess assay for NO$_2^-$ detection with the ion chromatography (IC) method. We also evaluated the possible interferences of H$_2$O$_2$ and peroxynitrite chemistry on the specificity of Griess assay by the addition of catalase as H$_2$O$_2$ scavenger. The specificity of the Indigo blue assay was investigated also by using scavengers of aqueous RONS (i.e. catalase, mannitol and sodium azide) in either real plasma activated aqueous solutions (PAW, PAPB) or in synthetic PAW (sPAW), i.e. chemical solution mimicking the PAW composition. Phenol degradation by TS electrospray was investigated in order to clarify the main chemical pathways in TS discharge activated solutions.

We tested two different Griess reagents: Griess 1 prepared according to [29] and Griess 2 (Nitrate/Nitrite Colorimetric Assay Kit). The comparison of the measured NO$_2^-$ concentrations by two different Griess assays (Griess 1 and Griess 2) and by ion chromatography showed no

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significant differences (**Figure 8** (left)). The results of the $NO_2^-$ detection by all three methods showed no significant difference between the samples with or without the added CAT. It seems that the reaction of Griess reagents with $NO_2^-$ is faster than the competing reaction of $H_2O_2$ with $NO_2^-$. Following these results, we confirmed that the Griess assay is a precise, reliable and suitable method for the nitrite detection in plasma activated solutions.
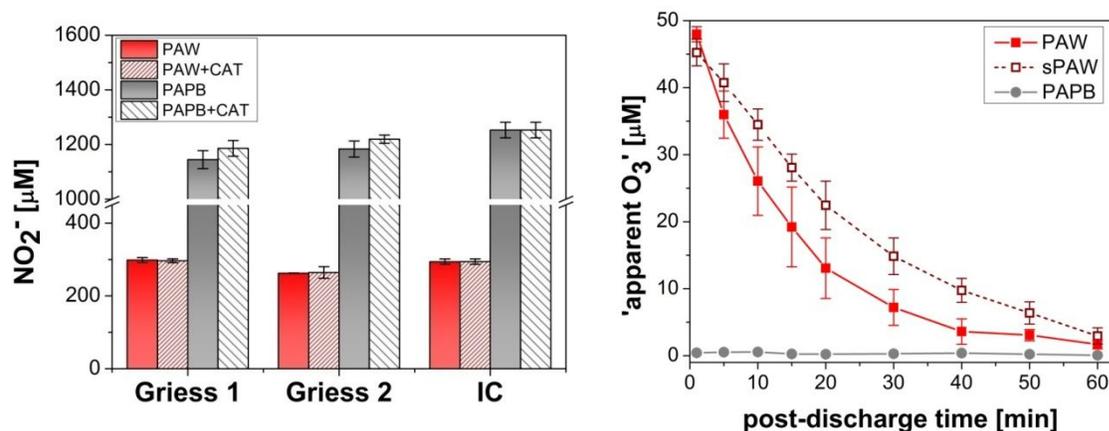


**Figure 8** Left: Comparison of the nitrite concentrations (mean +/- SEM, n = 3) in PAW and PAPB measured by Griess assay 1, Griess assay 2, and by ion chromatography (IC) (with (+CAT) and without added catalase (scavenger of $H_2O_2$)); right: Evolution of the 'apparent $O_3$' concentrations (mean +/- SEM, n = 3) in post plasma treatment time in PAW, PAPB and sPAW.

Interestingly, we previously detected by the Indigo blue method [30] the concentrations about 2.5 mg/L (52 μM) of dissolved $O_3$ in the PAW [31]. This was interesting, because TS discharge treatment decolorized the indigo blue dye even though there was no ozone detected in the gas phase as we showed. Therefore we verified the specificity of the indigo blue assay by using the simulated plasma activated water (sPAW), i.e. the solution with similar chemical composition and pH as our typical PAW prepared by air TS discharge. **Figure 8** (right) shows the so cold 'apparent $O_3$' detected by the indigo blue assay by the same manner in post plasma treatment time in PAW, PAPB and sPAW. The strong decolorization of the indigo blue dye was observed in PAW unlike in PAPB (0.1 M), where no decolorization was observed. The main difference between the PAW and PAPB was in the plasma induced chemical changes depending on pH of the plasma treated solution. In the PAPB, where pH remained non-acidified (~6.9), the concentrations of $H_2O_2$ and $NO_2^-$ were time-stable and the pH-dependent decomposition of $NO_2^-$ (*10, 11*) and ONOOH formation did not occur (*9*). On the other hand, in acidified PAW (pH 3.2) these reactions took place and furthermore the acidic decomposition of ONOOH (*15*) into $^•OH$ and $^•NO_2$ radicals occurred. The time evolution of 'apparent $O_3$' in the sPAW was observed identical to PAW, despite the fact, that this solution was not in any contact with plasma and therefore no $O_3$ could have been present. In sPAW, which contains only $H_2O_2$ and $NO_2^-$ at acidic pH, only ONOOH formation occurred, followed by its immediate decomposition (*15*) to $^•OH$ and $^•NO_2$ radicals. The results showed that the decolorization of the indigo blue dye in PAW and sPAW was almost the same despite no $O_3$ present in sPAW at all, and no $O_3$ was detected in the gas phase of TS discharge. We can thus assume that the Indigo blue decolorization in both these

solutions without ozone (PAW and sPAW) was most likely caused by the hydroxyl radical $^{\bullet}$OH formed as a decay product of ONOOH.

In order to further analyze aqueous RONS in plasma activated solutions, phenol was used as chemical probe to characterize the specific primary products of its degradation by the reactive species formed during the plasma treatment in aqueous solutions. Phenol is a suitable model of organic compound and its reactions give specific degradation by-products after reaction with $^{\bullet}$OH radical, $^{\bullet}$NO and $^{\bullet}$NO$_2$ radicals and especially with O$_3$.

**Table 2** Summary of the detected degradation by-products of phenol in PAW and PAPB.

| Phenol decomposition | | PAW | PAPB |
|---|---|---|---|
| **Phenol [µM]** | initial concentration | 500 | 500 |
| | after plasma treatment | 297.3 | 309.3 |
| | decomposed | 203 | 191 |
| **Hydroxylated products** | catechol benzoquinone hydroquinone hydroxybenzoquinone | 13 % (26 µM) | 11 % (21 µM) |
| **Nitrated products** | 4-nitrocatechol 2-nitrohydroquinone 4-nitrophenol 2-nitrophenol | 5 % (11 µM) | 1 % (2 µM) |
| **'Ring-cleavage by O$_3$' products** | *cis, cis*-muconic acid *cis, trans*-muconic acid | 0 | 0 |

The concentrations of residual phenol and its degradation products were analyzed using HPLC and the results are showed in **Table 2**. In PAW and PAPB solutions treated by the TS electrospray, only the hydroxylated degradation products and nitrated/nitrosylated by-products were detected, but no *cis, cis*-muconic acid (and its *cis, trans*-isomer) as a specific product of the ring cleavage of phenol by ozone. Contrary to the 'apparent O$_3$' detected by the Indigo blue assay, the results from the phenol degradation product analysis showed no muconic acid and therefore no dissolved O$_3$ in our air TS activated solutions. Moreover, the presence of hydroxylated and nitrated/nitrosylated by-products is an indirect evidence of the presence of peroxynitrous acid and its decay products in acidic pH – $^{\bullet}$OH and $^{\bullet}$NO$_2$ radicals (*15*), and also acidified nitrites (*10, 11*). These results support our previous evidence that the Indigo blue method is not specific to ozone detection in plasma activated solutions and strong peroxynitrite chemistry is responsible for the false ozone signal.

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# 6   List of author's publications

### i.   Publications in journals

- **Tarabová B.**, Lukeš P., Hammer M. U., Jablonowski H., von Woedtke T., Reuter S. and Machala Z.: Fluorescent measurement of peroxynitrite/peroxynitrous acid in cold air plasma treated aqueous solutions, *Phys. Chem. Chem. Phys.* **21,** pp. 8883-8896 (2019).

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- **Tarabová B.**, Lukeš P., Janda M., Hensel K., Šikurová L. and Machala Z.: Specificity of detection methods of nitrites and ozone in aqueous solutions activated by air plasma, *Plasma Process. Polym.* **15,** e1800030 (2018).

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    **Počet ohlasov na publikáciu –  111**

## ii.    List of relevant conference contributions

- Z. Machala, **B. Tarabová**, T. Vukušic, M. Janda, K. Hensel: *Plasma activated water chemical and antibacterial effects correlate with gaseous and aqueous RONS, plasma source, air and water flow conditions,* 24th International Symposium on Plasma Chemistry (ISPC 24), Napoli (Italy), June 9-14, (2019).
- **B. Tarabová**, P. Lukeš, E. Doležalová, R. Menthéour, Z. Machala, *Bactericidal effects induced by air transient spark with electrospray and/or PAW linked with RONS chemistry enhanced by other plasma agents*, 24th International Symposium on Plasma Chemistry (ISPC24), Napoli (Italy), June 9-14 (2019).
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