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Interpretation of fluorescent probe signals for the measurement of peroxynitrites in water solutions activated by air transient spark

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Abstract: We investigated the use of the fluorescent dye 2,7-dichlorodihydrofluorescein diacetate (H_2DCFDA) to detect peroxynitrites in air plasmas activated water (PAW) by transient spark discharge. The selectivity of the dye was investigated with the respect to peroxynitrites detection by using scavengers of reactive oxygen and nitrogen species (ROS and RNS) and dyes selective for others ROS/RNS. The aim of this study is to distinguish the part of the fluorescent signal that is due to the peroxynitrites presence in PAW.

Keywords: cold plasmas, plasma activated water, peroxynitrites, 2,7-dichlorodihydrofluorescein diacetate

1. Introduction

Studying bio-decontamination and other biomedical effects of cold plasmas is very important under wet conditions that are natural to cells. Cold plasmas generated in gases in contact with liquids produce the precursors of the reactive species followed by formation of the primary and secondary reactive species in the liquid through the gas-liquid interface, such as hydrogen peroxide H_2O_2, nitrites NO_2^-, nitrates NO_3^-, hypochlorite anion OCl^- and $OH^\cdot$, $NO^\cdot$ and $NO_2^\cdot$ radicals. Reactive oxygen and nitrogen species (ROS/RNS) lead to chemical changes (e.g. acidification) in water solutions and bactericidal effects on various microorganisms [1,2]. It has been shown that peroxynitrites chemistry in acidic environment significantly contributes to the bactericidal effects of PAW. Peroxynitrites are powerful oxidants that can oxidize many cellular components. The reactivity of peroxynitrites is pH-dependent and pK_a of $ONOO^-$/ONOOH system is 6.8. Peroxynitrites/peroxynitrous acid decay in acidic pH very fast to radicals:

$$ONOOH \leftrightarrow OH^\cdot + NO_2. \qquad (1)$$

These radicals induce strong cytotoxic effect and together with the acidic pH are responsible for the strong bactericidal effect. They even play a key role in the post-discharge bactericidal effect of PAW [3, 4]. The detection of peroxynitrites is generally difficult because of their high reactivity (very short life-time from milliseconds to seconds) and typically very low concentrations (~ nM). In [5], the correlation between the bactericidal effects of air transient spark induced in PAW with the presence of peroxynitrites detected by the 2,7-dichlorodihydrofluorescein diacetate dye (H_2DCFDA) was shown.

In this work, we focused our investigation on a specific detection of peroxynitrites by the fluorescent dye H_2DCFDA, and other dyes and different scavengers to decrease the cross-reactivity of other RONS with the dye in various water solutions activated by air plasmas.

2. Experimental methods

The experimental set-up of the water electrospray system is depicted in Fig. 1. DC transient spark discharge was generated in ambient air at atmospheric pressure in point-to-plane geometry. The water solutions were pushed through the high voltage electrode (hypodermic hollow needle with special cut tip) by a syringe pump. This allowed the water solutions to flow directly through the active zone of the discharge. Furthermore, due to the applied high voltage, we observed the effect of the electrospray – a finely nebulizing effect by tip enhanced electric field. This electrospraying enhanced the mass transfer of reactive oxygen and nitrogen species into the treated liquid solutions [6].

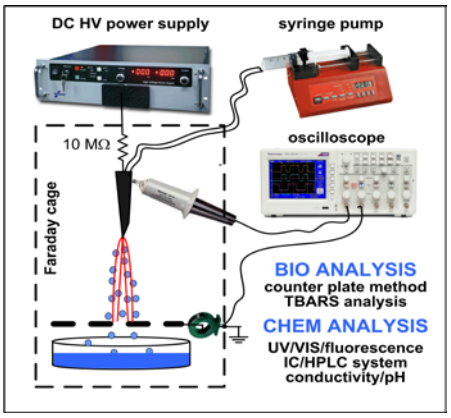

Fig. 1. Experimental set-up of air transient spark discharge with water electrospray

We used several working solutions differentiated with following parameters:

- **buffering activity:** buffered solutions – phosphate buffer (PB), phosphate buffered saline (PBS); non-buffered solutions – NaH_2PO_4 (low concentration mimicking tap water) and NaCl (physiological) solutions
- **pH:** acidic solutions (pH 3.3 – 3.5, prepared in H_3PO_4); neutral solutions (pH 6.8 – 7.4, prepared in PB/PBS)
- **content of Cl^-:** with Cl (NaCl, PBS); without Cl (NaH_2PO_4 solution, PB)

Working solution of the fluorescent 2,7-dichlorodihydrofluorescein dye was prepared according to assay described in [7]. To distinguish which part of the fluorescent signal is due to which reactive species, we tested several fluorescent dyes and also some scavengers of RONS:

- **dyes:** aminophenyl fluorescein (APF), described as specific for OCl^- and hydroxyphenyl fluorescein (HPF,) described as specific for $OH^\cdot$ radical
- **scavengers:** ebselen for $ONOO^-$, catalase for H_2O_2 and taurine for OCl^-.

We also used the following ROS/RNS as a chemicals by the bottle solutions: sodium peroxynitrite (Cayman Chemical), hydrogen peroxide and sodium hypochlorite (Sigma-Aldrich), and $OH^\cdot$ by the Fenton reaction.

3. Results and discussion

Air plasmas generate a great number of various reactive oxygen and nitrogen species in plasma treated liquids. These species could, besides peroxynitrites, lead to the cross-reactivities with the H_2DCFDA dye. The most distinguished cross-reactivities could be due to the expected high concentrations of H_2O_2 and OCl^- or high fluorescent response to $OH^\cdot$ [8].

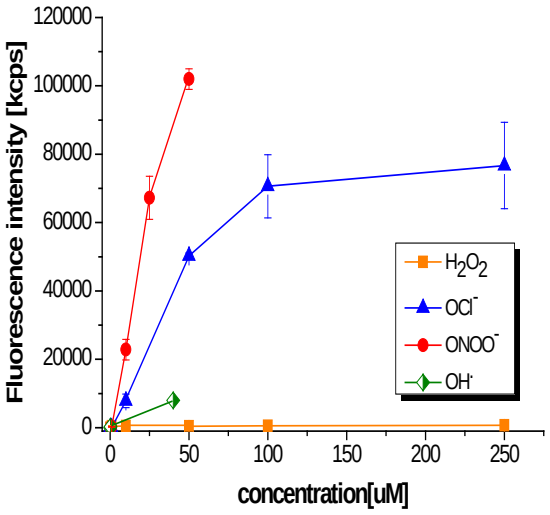

Fig. 2. Cross-reactivity of the fluorescent H_2DCFDA dye in dependence of oncentrations of various ROS/RNS, average +/- SD

In Fig. 2, the fluorescent responses of H_2DCFDA to these reactive species in PBS are shown. H_2DCFDA is most sensitive to $ONOO^-$ and then to OCl^-. Even the low concentration of peroxynitrites created higher fluorescence response than the same concentrations of hypochlorites. Fluorescent response of other species (H_2O_2, NO_x) was not significant, despite their typical concentrations in PAW treated by the same system are in ~ 100 μM. The response of H_2DCFDA to H_2O_2 was checked up to 5 mM concentration even in the acid environment and was found to be insignificant [5].

The fluorescent dyes APF and HPF were described as selective probes for highly reactive species – APF for OCl^- and HPF for $OH^\cdot$ radical [8]. APF measurement with the strongest response to OCl^- showed that lower concentrations of OCl^- were formed in our plasma treated PBS (data not shown). Furthermore, we found out experimentally that APF and HPF are not suitable for the acidic solutions. Therefore we could not detect the $OH^\cdot$ and OCl^- in non-buffered solutions.

Fig. 3 shows the time developments of the fluorescence singal of the H_2DCFDA with treated samples and various reactive species in different pH values of the solutions. We compared the response of the dye in neutral pH (treated PBS and solutions of RONS (10 μM) in pH 6.8-7.4) and in acidic pH (treated NaH_2PO_4 and solutions of ROS/RNS in pH 3.3-3.5). Firstly, the detection of the fluorescence signal development of H_2DCFDA dye with treated PBS and 10 μM solutions of $ONOO^-$ (pH 6.8) in time showed the similarity in the response of $ONOO^-$ solution and treated PBS. The OCl^- (pH 6.8) response is comparable with the control response. The response of the H_2DCFDA dye to $OH^\cdot$ radical produced by Fenton reaction is significantly lower than to the plasma treated samples, which is contrary to the study in [8]. The results in Fig. 3 also show that the response of H_2DCFDA is dependent on the pH of the solution. Knowing the pH of the working solution is important, because the chemistry pathways are different at different pH. [9]

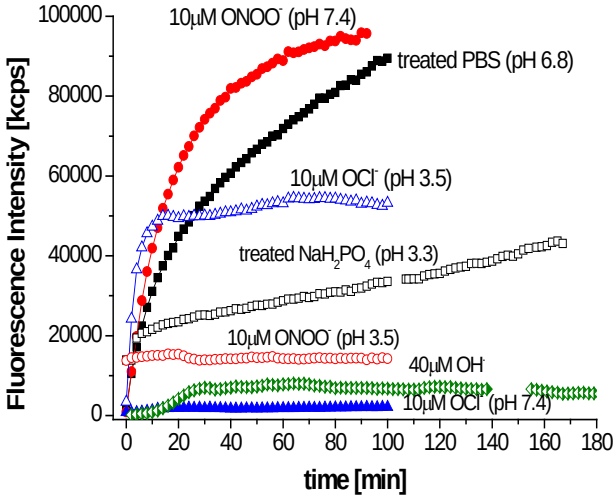

Fig. 3. Time development of the H_2DCFDA response to various ROS/RNS and PAW at different pH

To distinguish which part of the fluorescence signal is originated from which ROS/RNS, we worked with the specific scavengers of reactive species. As scavengers, we chose ebselen for $ONOO^-$, catalase for H_2O_2 and taurine for OCl^-. Firstly, we tested the scavengers' efficiency and their possible cross-reactivities with other reactive species. Ebselen is described as the most specific scavenger in literature with the second-order rate constant of 2.10^6 $M^{-1}s^{-1}$ of its reaction with $ONOO^-$ [10, 11]. We tested ebselen and observed that ebselen scavenged ~ 95% of $ONOO^-$. However, ebselen is able to scavenge OCl^-, too. Therefore ebselen cannot be used in plasma treated saline solutions and we need to find a more suitable scavenger for peroxynitrites scavenging in saline solutions. Also taurine seems not to be so specific for OCl^- and another scavenger e.g. hypotaurine should be tested. Data from scavenging efficiency are not shown.

The scavengers efficiency was also tested with the working solutions (NaH_2PO_4, PB and PBS) treated by transient spark discharge with respect to the H_2DCFDA signal (with or without added scavengers: catalase primarily scavenging H_2O_2 or ebselen primarily scavenging $ONOO^-$). The H_2DCFDA response to treated solutions without scavengers was the highest for NaH_2PO_4>PB>PBS (data not shown). In plasma activated NaH_2PO_4 solutions we observed the formation of hydrogen peroxide, nitrites NO_2^- and nitrates NO_3^- along with the acidification (pH 5 → 3.3). These conditions lead to the formation of peroxynitrites from H_2O_2 and NO_2^- [3,4]:

$NO_2^- + H_2O_2 + H^+ \rightarrow ONOOH + H_2O$ (2)

In PBS solution, the signal is not only due to the peroxynitrites, but also to OCl^-. By addition of ebselen into the treated samples we observed a decrease of fluorescence signal. We expected that the missing part of the signal in NaH_2PO_4 and PB is due to the scavenging of peroxynitrites, but in PBS ebselen scavenged both peroxynitrites and hypochlorites. The rest of the signals in all samples are probably due to the small cross-reactivities of other reactive species and autofluorescence. By addition of catalase into the treated NaH_2PO_4 solution we observed a significant decrease of the H_2DCFDA fluorescence intensity. We expect that catalase scavenged hydrogen peroxide needed for the peroxynitrites formation (2) and therefore no or minimal concentrations of $ONOO^-$ were created. In this case, the decrease of the signal is due to the absence of peroxynitrites in the treated solution. This result supports the fact that peroxynitrite chemistry is continuing as well as that H_2DCFDA can be used for peroxynitrites detection if the ROS/RNS are limited. In case of added catalase into the PBS, we observed no change in the fluorescence intensity. This can be due to the fact, that OCl^-/HOCl imposes a rapid inactivation of catalase [12].

4. Summary

Peroxynitrites are postulated to be the key antimicrobial agent in plasma activated water. In this work, we showed a new approach to the peroxynitrites detection in air plasmas activated water solutions. We found out that the chemistry pathways at different pH took place in different ways. Because of the presence of various ROS/RNS in plasma activated water solutions, the possible cross-reactivities and short half-lifes make the detection of peroxynitrites difficult. Nevertheless, we demonstrated that at least in plasma activated solutions with no Cl^- present and therefore no hypochlorite anions created, the H_2DCFDA fluorescence signal is mostly due to peroxynitrites and this fluorescent spectroscopic method seems to be promising under considering some limitations (plasma treatment condition, type of the discharge or composition of the working solutions).

5. Acknowledgement

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