**Reactions of nitroxide radicals in aqueous solutions exposed to non-thermal plasma: Limitations of spin trapping of the plasma induced species**

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**Abstract**

Low temperature (‘cold’) atmospheric pressure plasmas have gained much attention in recent years due to their biomedical effects achieved through the interactions of plasma-induced species with the biological substrate. Monitoring of the radical species in an aqueous biological milieu is usually performed *via* electron paramagnetic resonance (EPR) spectroscopy using various nitrone spin traps, which form persistent radical adducts with the short-lived radicals. However, the stability of these nitroxide radical adducts in the plasma-specific environment is not well known. In this work, chemical transformations of nitroxide radicals in aqueous solutions using a model nitroxide 4-oxo-TEMPO were studied using EPR and LC-MS. The kinetics of the nitroxide decay when the solution was exposed to plasma were assessed, and the reactive pathways proposed. The use of different scavengers enabled identification of the types of reactive species which cause the decay, indicating the predominant nitroxide group reduction in oxygen-free plasmas. The 2H adduct of the PBN spin trap (PBN-D) was shown to decay similarly to the model molecule 4-oxo-TEMPO. The decay of the spin adducts in plasma-treated solutions must be considered to avoid rendering the spin trapping results unreliable. In particular, the selectivity of the decay indicated the limitations of the PTIO/PTI nitroxide system in the detection of nitric oxide.

**Introduction**

Non-thermal, or low temperature plasmas (LTP) have attracted increased attention over recent years, largely due to their potential biomedical applications [1-3]. These plasmas, operated at atmospheric pressure, can be applied directly to a tissue without thermally damaging it. Low-temperature plasmas are also used for surface and material modification [4], catalysis [5,6], removal of VOC from air [6], waste water treatment [7], etc.

Materials treated with LTPs are exposed to a variety of reactive species, including molecular, atomic, radical and ionic compounds [8,9]. These species may be responsible for the LTP’s reported anti-cancer, anti-bacterial and anti-viral activity [1,3,10]. Further development of LTP applications thus requires chemical characterisation of the plasma-exposed liquids. This can be achieved by a range of analytical techniques such as colourimetry, liquid chromatography [11], mass spectrometry [12], flow cytometry [13], electron paramagnetic resonance spectroscopy [9,13,14]. Detection of highly reactive plasma-induced free radicals in the liquid media exposed to plasma jet is of particular interest.

Free radical species are generally short-lived, and in most cases cannot be monitored directly in a liquid solution. The technique used in research is spin trapping of the radicals and subsequent EPR analysis of the more stable (and thus detectable) spin adducts formed [15]. The organic reagents used in this method (spin traps) are usually nitronesor chelated metal complexes, allowing detection of •OH, •OOH, •NO and carbon-centred radicals [9,16-18]. An example of a spin trapping reaction with *N*-t*ert*-butyl--phenylnitrone (PBN) spin trap is shown in Scheme 1.

Tresp *et al.* and Tani *et al.* described the measurement of the hydroxyl radical •OH, superoxide/hydroperoxyl radical O2•-/•OOH in aqueous solutions using DMPO, BMPO and other spin traps [14,19]. Various radical species generated by plasma exposure were detected and quantified by Takamatsu *et al.* in buffered aqueous solutions [9]. For a kHz frequency-driven parallel field plasma jet, some of the radicals (•OH, •H and •OOH) in liquids treated with LTP were shown to originate in the gas phase and delivered directly into the liquid [18]. Other radicals were shown to be formed as a result of secondary reactions inside the liquid medium, *e.g.* from dissociation of plasma-induced peroxynitrite anion ONOO- [11]. Uchiyama *et al.* performed quantitative studies of •OH, •H and O2•- (•OOH) radicals in plasma-treated liquid samples using DMPO, PBN and M4PO nitrone spin traps. The authors also performed •NO radical trapping using carboxy-PTIO spin trap [13].

However, spin adducts show only moderate stability, and decay over extended periods of time [20].In plasma-treated liquids, spin adducts could be efficiently degraded by the reactive species. For instance, oxidative degradation was demonstrated for various organic compounds [7,21] in aqueous solutions with ignited plasma. More recently, amino acids modification was observed in plasma-treated solutions [22]. The degradation of spin adducts in plasma-treated liquids can thus strongly affect experimental results of spin trapping; this effect however is often overlooked.

In the present work, an experimental study of the decay of organic nitroxides in plasma-treated aqueous solutions is presented. Using a model nitroxide compound 4-oxo-2,2,6,6-tetramethylpiperidine 1-oxyl (4-oxo-TEMPO), we demonstrate a non-zero order decay kinetics. LC-MS analysis of reaction mixtures after the plasma exposure reveal that the main product results from the reduction of the nitroxide group. By introducing different scavengers of the reducing or oxidising species induced by the plasma, the mechanism of the nitroxide decay during plasma exposure was investigated. The plasma-induced decay of the model nitroxide was compared to that of a spin adduct PBN-D. The stability of nitronyl nitroxide radical 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO), which is used for the detection of nitric oxide, and the product of its reaction with •NO, imino nitroxide 2-phenyl-4,4,5,5-tetramethylimidazoline 1-oxyl (PTI) was also investigated.

**Experimental**

**Plasma setup.** The plasma was ignited inside a quartz tube (4 mm ID and 6 mm OD, 100 mm length) surrounded by copper electrodes (10 mm width) separated by 20 mm. The plasma was sustained using a sinusoidal voltage waveform (PVM500 Plasma Resonant and Dielectric Barrier Corona Driver power supply (Information Unlimited)). A high-voltage probe (Tektronix P6015A) and current probe (Ion Physics Corporation CM-100-L) were used with a Teledyne LeCroy WaveJet 354A oscilloscope to measure time resolved current and voltage. Voltage and frequency were kept constant throughout all experiments at 18.3 ± 0.2 kV (peak-to-peak) and 24.9 kHz, respectively. The return current values were between *ca.* 4 and 7 mA (for water vapour-saturated and dry He, respectively). A more detailed plasma characterisation (voltage and current waveforms, OES measurements of the plasma between the electrodes) is described elsewhere [18].

The plasma was operated with a helium feed gas (A Grade, 99.996%) with oxygen (Zero Grade, 99.6%), air and water admixtures controlled by mass flow controllers (MFCs) (Brooks Instruments and Brooks Instruments 0254 microcomputer controller). All experiments were carried out with a total flow of 2 slm of the feed gas. The percentage of the O2 (Zero Grade, 99.6%) or air admixture is quoted in vol%. Water-saturated helium was produced by bubbling dry helium through a water-filled Drechsel flask at 20 °C. The relative humidity was determined by weighing the flask before and after the experiment and comparing the data with the available literature values [23]. The experimental setup was positioned inside a large Faraday cage.

**Analysis.** Electron paramagnetic resonance (EPR) measurements were carried out on a Bruker EMX Micro EPR spectrometer equipped with an EMX high sensitivity probehead. The EPR analysis parameters were as follows: frequency 9.83 GHz, power 3.17 mW, modulation frequency 100 kHz, modulation amplitude 1 G, time constant 40.96 ms, conversion time 40 s, number of scans 5, sweep width 100 G. For the measurements, a sample (*ca.* 50 L) was contained in glass capillary tubes (80 x 1 mm) purchased from Marienfeld Laboratory Glassware. Calibration of EPR double integral intensity was performed using aqueous solutions of a stable radical (TEMPO) in a range of concentrations 2-200 M. After each plasma exposure experiment, the samples were immediately placed in a capillary tube. The overall time between the exposure and recording the spectrum was 1 minute in all cases. EPR spectra simulations were performed on NIH P.E.S.T. WinSIM software ver. 0.96 [24].

The *g*-factor values were determined using a 2,2-diphenyl-1-picrylhydrazyl (DPPH) standard (see Supporting Information) as 2.0057, 2.0066 and 2.0058 for 4-oxo-TEMPO, PTIO and PBN-H, respectively.

The pH of the solutions was monitored using the Hydrion Insta-check 0-13 pH test paper obtained from Sigma. No change in pH values was observed when samples containing aqueous solutions of 4-oxo-TEMPO, PTIO or PBN were exposed to He, He + 0.5% O2, He + 0.5% air or He + 100% H2O vapour plasma.

For nitrite measurements, 50 L of a plasma-treated sample were mixed with 350 L of H2O and 400 L of the Griess reagent. Samples were incubated for 5 minutes in the dark at room temperature and analysed by UV.

UV-Vis measurements were performed on a UV-1800 Shimadzu UV-Vis Spectrophotometer with Optical Glass High Precision Cells (10 mm light path) provided by Hellma Analytics. Aqueous solutions of 4-oxo-TEMPO with hydroquinone and benzoquinone were diluted 1:50 with H2O prior to UV-Vis analysis. Nitrite calibration was performed using 400 L of Griess reagent with 400 L of aqueous NaNO2 solutions of different concentrations (3-100 M) using the signal intensity at 526 nm.

LC-MS analysis was performed on a Dionex UltiMate 3000 HPLC system coupled to a Bruker HCT Ultra ETD II ion trap mass spectrometer with an ESI interface. Analytes were detected in the positive ion mode using the following MS parameters: capillary voltage 4500 V; nebulizer gas 50 psi, drying gas flow 9.0 L/min; drying temperature 350 oC and acquired in MS mode over the scan range m/z 50-400. Chromatographic separation was carried out using a Waters SymmetryShield RP18 5 m column, 4.6x250 mm. The binary mobile phase was composed of (A) water + 0.01% (v/v) formic acid and (B) acetonitrile. The flow rate was 1.0 mL/min with the outlet split to give a flow into the ESI source of *ca.* 0.3 mL/min. The calibration was performed with aqueous solutions of 1-hydroxy-2,2,6,6-tetramethylpiperidine (4-oxo-TEMPOH) and 4-oxo-2,2,6,6-tetramethylpiperidine (4-oxo-TEMP) of 10-400 M concentrations.

The concentrations of all reactive species in the liquid samples are quoted after correction for solvent evaporation. The conversion values of 4-oxo-TEMPO and PBN were calculated as follows:

, where *C* is the concentration of the respective compound.

**Plasma exposure experiments.** In a typical experiment, a liquid sample (100 L) was placed in a well on top of a glass stand inside the reactor. The distance from the nozzle to the sample was 10 mm. The distance between the electrodes was 20 mm. The reactor was flushed with the feed gas for 20 s and then exposed to plasma for 60 s.

PBN spin trapping experiments were performed as follows. 200 L of a 100 mM solution of PBN in H2O were exposed to a He plasma with 50% D2O vapour saturation using a split helium flow (*i.e.*, by mixing dry helium with D2O-saturated helium 1:1 to allow a total flow of 2 slm). After the exposure the aqueous sample was collected into a sample vial and frozen in a saline ice bath (*ca.* -20 oC). The setup was flushed with dry He gas (5 slm, 10 min). The sample was then thawed and an aliquot A (*ca.* 40 L) was analysed by EPR immediately before the exposure to another plasma (He, He + 0.5% O2 or He + 100% H2O). A 100 L aliquot B of the remaining solution was exposed to this second plasma and analysed by EPR after the exposure. To correct for the radical adduct decay occurring regardless of plasma, aliquot A was left at room temperature for the duration of the experiment with aliquot B, after which aliquot A was again analysed by EPR. The procedure was repeated for each of the plasma exposures (He, He + 0.5% O2 and He + 100% H2O).

For the nitroxide decay experiments, a solution of 4-oxo-TEMPO (9 M – 50 mM), PTIO and PTI separately or combined (200 M each), PBN-D (*ca.* 9 M) were exposed to the plasma. Hydroquinone (scavenger for oxidising species), *p*-benzoquinone (scavenger for reducing species) or glycine (more selective scavenger for the hydroxyl radical) were added to a 200 M solution of 4-oxo-TEMPO in 100 eq (20 mM) prior to plasma treatment, where stated. PBN (*ca.* 130 mM) was added to a 9 M solution of 4-oxo-TEMPO where stated.

For the nitric oxide trapping experiments, a mixture of 50 L of MGD sodium salt monohydrate (10 mM) was added to 50 L of FeSO4·7H2O (2 mM). The solutions were degassed with argon prior to experiments. After the resulting solution was exposed to plasma, 25 L of 20 mM Na2S2O4 were added (to reduce any chelated Fe3+ which was formed during the experiment, back to Fe2+ [25]). The sample was analysed by EPR.

Each data point corresponds to a separate reaction run.

**Error assessment.** The evaluation of the experimental reproducibility of the results was performed as follows. 4-Oxo-TEMPO and PTIO decay experiments were repeated 10 times. In these experiments, 100 L samples of a solutions 4-oxo-TEMPO or PTIO were exposed to He, He + 0.5% air or He + 0.5% O2 plasma and the concentrations of the compounds after the exposure were compared. The determined standard deviations are shown as error bars on respective figures (see also Supporting Information, Table S1). The results were in good accordance with the radical trapping error evaluation described elsewhere [18].



**Scheme 1. The formation of spin adducts with PBN spin trap.**

**Results and discussion**

In LTP-exposed liquid samples, the highly reactive species produced by the plasma participate in chemical reactions occurring in the liquid. Both spin traps and spin adducts may be affected by these reactions. The decomposition of spin traps by reactive species in LTP-treated solutions is often negligible as spin traps are used in a large excess in the reaction system. The spin adducts (nitroxides), on the other hand, can be susceptible to decay *via* reactions with plasma-generated species.

In the context of LTPs, nitroxides are used not only as spin adducts but also to monitor O3, atomic oxygen and 1O2 *via* their reaction with the parent piperidines e.g., 2,2,6,6-tetramethylpiperidine (TEMP) [18,26]. However, we observed evaporation of 2,2,6,6-tetramethylpiperidine 1-oxyl (TEMPO) from dilute aqueous solutions exposed to a 2 slm gas flow. After 80 s of exposure to the helium flow at room temperature, the concentration of TEMPO in H2O decreased from 200 M to *ca.* 120 M as determined by EPR (data not shown). This, in addition to the limited selectivity, indicates limited applicability of TEMP and TEMPO to quantitative studies of plasma-generated species in solutions. Therefore, nitroxide stability in plasma-exposed aqueous solutions was investigated in this work using less volatile 4-oxo-TEMPO as a model stable free radical. Importantly, the control experiments revealed no evaporation of 4-oxo-TEMPO and other radical compounds used in this work, such as radical adducts and nitronyl and imino nitroxides (*vide infra*).

**4-oxo-TEMPO decay in aqueous solutions exposed to plasma.** To assess the stability of nitroxides in plasma-treated liquids, aqueous 4-oxo-TEMPO solutions of different concentrations (50 M to 50 mM) were exposed to a plasma jet. We acknowledge that the reactivity of 4-oxo-TEMPO and spin adducts may differ, however the comparison of the two can be performed under the assumption that their chemistry is dominated by the reactions of the nitroxide group.

An in-house built reactor (described elsewhere [18]) was used to allow control of the ambient atmosphere. The gaseous phase inside the reactor was comprised of the feed gas components and evaporated solution from the exposed aqueous sample. This limited the main plasma-induced reactive species in the liquid to hydrogen peroxide (H2O2), radical species (•OOH, •OH, •H) and O3/O. The solutions were then analysed by EPR spectroscopy.

The results clearly demonstrated that aqueous 4-oxo-TEMPO decayed when treated with plasma (Figure 1). Moreover, the initial decay rate was dependent on the nitroxide concentration. At low initial concentrations of 4-oxo-TEMPO (see expansion in Figure 1), the changes in initial decay rate approached pseudo-first order kinetics (see SI, Table S2). At higher concentrations, the effective kinetic order became fractional. The kinetic behavior of 4-oxo-TEMPO degradation was thus complex.



**Figure 1. 4-oxo-TEMPO decay in aqueous solutions of different concentrations exposed to helium plasma. Initial concentrations of the solutions:  50 mM;  20 mM;  2 mM;  200 M;  100 M;  50 M. The error bar in this and further graphs represents the average error in 10 repetitions of the experiment under identical conditions (Table S1).**

**Decay pathways and products.** The decay of LTP-exposed 4-oxo-TEMPO solutions with various admixtures is shown in Figure 2. The 200 M concentration of 4-oxo-TEMPO was chosen as it gave high conversion within a relatively short exposure time.



**Figure 2. 4-oxo-TEMPO decay in aqueous solutions exposed to different plasmas. Hydroquinone (HQ) and *p*-benzoquinone (BQ) were added in 100 eq concentrations prior to plasma exposure.**

It was observed that 4-oxo-TEMPO decayed at a similar rate with both a pure helium feed gas and helium with a 0.5% oxygen admixture. For water vapour-saturated helium, both the initial decay rate and the conversion of 4-oxo-TEMPO decreased. In our previous findings, the presence of large quantities of water vapour in the feed gas resulted in substantially reduced amounts of radicals in the liquid sample, whereas the concentration of H2O2 induced into the liquid sample was high [18]. The control experiments in which hydrogen peroxide was added to a solution of 4-oxo-TEMPO in concentrations up to 100 eq (20 mM) did not result in a radical decay. This suggested that 4-oxo-TEMPO decay was due to other plasma-induced species, *e.g.* radicals.

In order to further elucidate the mechanism of 4-oxo-TEMPO decay, the post-reaction solutions containing 4-oxo-TEMPO decay products were analysed by LC-MS (Figure 3). The LC-MS calibration was performed using 4-oxo-TEMPOH and 4-oxo-TEMP solutions of various concentrations (see Experimental section and SI, Figure S1 (b,c), Table S3).



**Figure 3. Concentrations of 4-oxo-TEMPOH and 4-oxo-TEMP in aqueous solutions exposed to (a) He and (b) He + 0.5% oxygen plasma as determined by LC-MS. 4-oxo-TEMPO decay (dashed) was determined by EPR.**

The results of the time-resolved product analysis demonstrated that 4-oxo-TEMPO decay during pure helium plasma exposure was quite selective. Here, the dominating product was 4-oxo-TEMPOH (possibly formed through direct reduction of 4-oxo-TEMPO by hydrogen atoms; Scheme 2) with the yield reaching 75% based on the initial 4-oxo-TEMPO concentration (Figure 3a).



**Scheme 2. Some of the possible 4-oxo-TEMPO transformation pathways in reactions with plasma-induced radical species.**

The amount of 4-oxo-TEMPOH produced was substantially lower in the case of He + O2 plasma (*ca.* 15 M, 7% yield). The complexity of the chemical reactions of 4-oxo-TEMPO in water under plasma exposure likely increased in the presence of molecular oxygen in the plasma feed gas. The formation of reactive oxygen species such as O3 and atomic oxygen [27] may have reduced the amount of •H radical [18] which is responsible for the reduction of 4-oxo-TEMPO to the corresponding hydroxylamine (4-oxo-TEMPOH).It was observed that under these conditions the concentration of 4-oxo-TEMPOH slightly decreased after an initial increase (Figure 3b), suggesting that this product underwent further transformation. The remaining mass balance was thus ascribed to possible formation of oxidation products, *e.g.* oxoammonium compounds or ring opening products (Scheme 2).

With the H2O-saturated feed gas, both the conversion of 4-oxo-TEMPO and the yields of the reduction products were lower than those with He (SI, Figure S2). However, this exposure was selective towards 4-oxo-TEMPOH and 4-oxo-TEMP formation (yields 72% and 26%, respectively). In all cases the amount of formed 4-oxo-TEMP was similar, suggesting it was at least partially *via* other reactions, not involving •H or •OH radicals.

The identification of the reactive species responsible for nitroxide decay was performed by introducing various scavengers in the aqueous solutions of 4-oxo-TEMPO prior to plasma treatment. Hydroquinone (HQ) and *p*-benzoquinone (BQ) were introduced as scavengers for oxidising and reducing species, respectively. Glycine was added as a more selective scavenger of the •OH radical (e.g., glycine does not readily react with ozone). The results are presented in Figures 2 and 4. It must be noted that no decay of 4-oxo-TEMPO due to the addition of the scavengers was observed within the timescale of the experiments.



**Figure 4. 4-oxo-TEMPO conversion in aqueous solutions with added scavengers exposed to different plasmas. Initial 4-oxo-TEMPO concentration was 200 M. Hydroquinone (HQ), *p*-benzoquinone (BQ) or glycine were added in 100 eq concentrations (20 mM) prior to plasma treatment. Exposure time 60 s.**

Addition of glycine did not affect the extent of 4-oxo-TEMPO decay (Figure 4). A possible explanation could be that in this case 4-oxo-TEMPO decays by recombination with carbon-centred glycyl or aminyl radicals (formed by a reaction of glycine with the hydroxyl radical) [28]. However, mass spectrometry analysis gave no evidence for either 4-oxo-TEMPO-glycyl or aminyl adduct formation, while 4-oxo-TEMP and 4-oxo-TEMPOH were detected (Figure S3). Additionally, the reported rate of the reaction of 4-oxo-TEMPO with hydroxyl radical was higher than that for glycine [29,30]. In other words, 4-oxo-TEMPO is an excellent scavenger of radical species, and introduction of other scavengers even at high concentrations does not necessarily result in ‘protection’ of 4-oxo-TEMPO.

With added BQ (reducing species scavenger), 4-oxo-TEMPO conversion decreased from *ca.* 94% to 33% upon exposure to the helium plasma. This is in agreement with the suggested reduction of the nitroxide group by •H radical or solvated electrons (which would be partially scavenged by BQ). A decrease in 4-oxo-TEMPO conversion was also observed for the H2O-saturated He plasma treatment. The addition of HQ (oxidising species scavenger) reduced the conversion of 4-oxo-TEMPO exposed to the He + 0.5% O2 plasma from *ca.* 87% to 58%. The fact that the decay was in these cases reduced but not completely suppressed suggests the limited effectiveness of scavenging plasma-induced species. Indeed, the UV spectroscopy analysis of the post-exposure aqueous solutions with added BQ and HQ admixtures revealed that the scavengers remained largely intact (SI, Figure S4).

These data obtained suggest the following pathways for the radical decay: 1) •H driven reduction by He plasma (with and without H2O vapour); 2) predominantly oxidation when exposed to the helium with oxygen plasma. This radical decay pathway is in agreement with the literature on related processes. Chemical transformations of TEMPO through reactions with *e.g.* hydrogen and hydroxyl radicals were described for photochemical systems by Kudo *et al.* [31] and Marshall *et al.* [32]. The hydroxyl radical was generated either by photolysis of H2O2 or photochemically (TiO2) from H2O molecules. The authors report formation of the reduced TEMPO derivatives such as hydroxylamines and piperidines. This occurs either *via* hydrogen atom reduction of the nitroxide group, or the initial formation of the aminyl radical (Scheme 2). We acknowledge that in the employed plasma system, unlike photochemical mixtures, a variety of charged species may also be present. Reaction of nitroxides with superoxide is not included in Scheme 2; nitroxides act as catalysts for superoxide dismutation and are thus not degraded by superoxide [33].

**Decay of the radical adducts of PBN.** PBN spin trap is oftenused to detect radicals in liquid media. The plasma-induced decay of its spin adducts was therefore investigated. The exposure of a PBN spin trap solution to plasma yields a PBN-H spin adduct upon exposure to pure He plasma [18] (see Scheme 1). If the solution containing this adduct is further exposed to the same or another plasma, some of the formed radical adduct will decay, but more will be formed through trapping of the newly generated radicals (see also SI, Table S4).

The use of isotopically labeled water D2O made it possible to distinguish between the original spin adduct and that formed during the secondary exposure. A sample of 100 mM aqueous (H2O) solution of PBN was exposed to a He plasma partly saturated with D2O vapour (*ca.* 50% relative humidity). The EPR analysis of the resulting mixture of PBN-H and PBN-D radical adducts (Table 1) was used to separately monitor each adduct as they have distinctly different signals (Figure 5a). This mixture was further exposed to different (non-D2O containing) plasmas. As no more PBN-D adduct is formed during this secondary exposure, its concentration was used to directly assess the rate of its plasma-induced decay. The limited stability of the PBN-D adduct in the absence of plasma was taken into account by monitoring the decay of a control sample.



**Figure 5. Typical experimental and simulated EPR spectra of (a) PBN-H and PBN-D radical adducts mixture and (b) PTIO and PTI (nitronyl nitroxide and corresponding imino nitroxide) mixture. PBN-H: aN = 16.6 G, aH = 10.8 G (×2); PBN-D: aN = 16.7 G, aH = 10.8 G, aD = 1.6 G; PTI: aN1 = 9.9 G, aN2 = 4.4 G; PTIO: aN = 8.2 G (×2).**

**Table 1. Decay of PBN-H and PBN-D in aqueous solutions exposed to different plasmas. 100 mM solution of PBN was used to form PBN-D which was further employed in decay experiments. All solutions contained unreacted PBN (*ca.* 130 M). Plasma exposure time 60 s.**

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Entry | Plasma feed gas | Concentration before exposure (M) | |  | Concentration after exposure (M) | | Concentration of PBN-D in a control (untreated) sample (M) | PBN-D conversion due to plasma exposure (%) |
| PBN-D | PBN-H |  | PBN-D | PBN-H |
| 1 | He | 9.3 | 2.3 |  | 4.6 | 8.4 | 9.2 | 49 |
| 2 | He + 0.5% O2 | 8.7 | 2.5 |  | 3.5 | 1.7 | 8.2 | 54 |
| 3 | He + 100% H2O | 8.4 | 2.0 |  | 5.6 | 5.2 | 8.4 | 33 |

**Table 2. Decay of 4-oxo-TEMPO in aqueous solutions exposed to different plasmas. All solutions contained PBN (*ca.* 130 M). Plasma exposure time 60 s.**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Entry | Plasma feed gas | 4-oxo-TEMPO concentration before exposure (M) | 4-oxo-TEMPO concentration after exposure (M) | 4-oxo-TEMPO conversion (%) |
| 1 | He | 9.6 | 5.2 | 46 |
| 2 | He + 0.5% O2 | 9.6 | 5.1 | 47 |
| 3 | He + 100% H2O | 9.6 | 6.1 | 37 |

As a result of the secondary exposure, the PBN-D adduct decayed with *ca.* 50% conversion in the He plasma without additives or with 0.5% oxygen (Table 1, entries 1-2). The exposure to the H2O-saturated He plasma resulted in a lower PBN-D decay (33%, entry 3), as expected. These data unambiguously demonstrate that spin adducts are prone to decay in plasma-exposed aqueous media. The concentrations of spin adducts therefore include contributions from both radical trapping and adduct decay. Both processes will depend on the nature and concentration of reactive species, i.e. the specific spin adduct, components of the liquid, plasma feed gas, temperature, etc. This may have a large impact on the detected amount of the radical adduct, and hence quantitative comparison of spin adducts in plasma-exposed samples should be considered with caution.

4-oxo-TEMPO showed a much higher decay (>99% conversion) upon exposure to plasma at concentration corresponding to that of PBN-D in the same experiments (*ca.* 9 ). However, when PBN was added to 4-oxo-TEMPO solutions prior to plasma exposure, the conversion of 4-oxo-TEMPO was drastically reduced (Table 2). We infer that the unreacted nitrone acts as a scavenger, protecting the nitroxides from decay by the plasma-induced species. Under identical conditions, the conversion of 4-oxo-TEMPO was very similar to that of PBN-D adduct (*e.g.,* 46% and 49%, respectively, when exposed to the He plasma). These data support the assumption that the main decay pathways of both TEMPO and PBN-H/D are defined by the reactivity of the nitroxide group. This once again emphasised that the radical adducts formed in the spin trapping reactions are highly prone to decay by the same plasma-generated species which led to their formation.

**Spin trapping with PTIO.** The relative reactivity of nitroxides with the plasma-generated species is important for radical detection systems comprised of several nitroxides. For example, nitronyl nitroxides such as 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO) are used in chemical and biological systems as spin traps for nitric oxide •NO [16,25,34]. The reaction of PTIO with •NO leads to the formation of the imino nitroxide 2-phenyl-4,4,5,5-tetramethylimidazoline 1-oxyl (PTI) as shown in Scheme 3 [35]. Here, the difference between the nitrone spin trapping and the use of PTIO is that both the product PTI and the starting compound PTIO are nitroxides and both have EPR signals (Figure 5b).



**Scheme 3. The formation of imino nitroxide PTI *via* reaction of nitronyl nitroxide PTIO with nitric oxide** •**NO.**

Recently, Uchiyama *et al.* reported the use of PTIO derivatives to detect •NO radical generated by the interaction of plasma with ambient air and delivered to the aqueous media [13]. The authors suggested that the absence of the imino nitroxide signal was a result of low •NO concentration and thus low (undetectable) imino nitroxide yield. We have assessed the stability of PTIO and its product PTI in aqueous solutions exposed to plasma. For this purpose, PTI was synthesised by reacting PTIO with NaNO2 in methanol.The resulting solution was further treated with PbO2 to achieve high yields of the PTI radical (see SI).

The •NO radical (along with other reactive nitrogen species) was generated in the plasma by admixing air in the feed gas. The exposure of 200 M solution of PTIO in H2O to the He + 0.5% air plasma and subsequent EPR analysis showed decay profiles resembling that of 4-oxo-TEMPO (Figure 6a, see also Figure S5). Similar profiles were obtained for both He and He + 0.5% O2 plasmas (SI, Figure S5). However, the exposure of the aqueous PTI solution to plasma has shown that PTI was more prone to decay than PTIO *e.g.* with oxygen-containing plasma (Figure S5b). Importantly, PTI was not formed when PTIO was exposed to the air-containing plasma. Moreover, it is seen that PTI was partially converted back to PTIO, possibly *via* reaction with the oxygen species created by the plasma (Figure 6a).



**Figure 6. (a) PTIO and PTI decay by He + 0.5% air plasma, (b) PTI and PTIO mixture decay by He plasma in aqueous solutions. The initial concentration of each compound in all experiments was 200 M.**

The trapping of •NO by PTIO yields a mixture of PTIO and PTI. Hence, the stability of these species upon plasma exposure was assessed when both were present in a solution, each at 200 M concentration). The results indicated that PTI decay was enhanced in the presence of PTIO when the solution was exposed to *e.g.* He plasma (Figure 6b). For instance, the concentration of PTI after 30 seconds of plasma exposure was *ca.* 20 M (compared to 75 M in the absence of added PTIO; Figure S5). This was probably due to reactions of PTI with the products of PTIO degradation. At the same time, the decay of PTIO was hindered in the presence of added PTI. Two factors are probably responsible for this. First, PTI acted as a scavenger (similarly to PBN, *vide supra*). Secondly, some PTI probably underwent transformation into PTIO (in He + O2 or He + air plasmas). Overall, PTI decays considerably faster than PTIO in plasma-exposed solutions. Therefore, the absence of PTI in plasma-exposed PTIO solutions does not necessarily imply the absence of •NO radical, and one has to use this method with caution.

It is worth noting that the use of a different spin trap for •NO (a chelate complex (MGD)2-Fe2+ [16,25]) led to the formation of a (MGD)2-Fe2+-NO adduct upon exposure to He + 0.5% air plasma (SI, Figure S6, Table S5). However, this method is not specific for the •NO radical. The oxidation of the metal centre (Fe2+  Fe3+) followed by a reaction with nitrite anion NO2- also yields (MGD)2-Fe2+-NO species [36]. Hence, (MGD)2-Fe2+ cannot differentiate between •NO and NO2- (the measured concentration of nitrite in a solution after exposure to He + 0.5% air plasma was *ca.* 200 M). When PTIO was exposed to He + air plasma at the same concentration as (MGD)2-Fe2+, no PTI was detected with partial PTIO decay (Table S6), but this does not necessarily signify the absence of •NO in the liquid phase [16], and even more so in solutions treated by plasma as described above. As the use of either of these techniques for the detection of •NO radical has limitations, accurate measurement of •NO in plasma-exposed liquids is challenging.

**Conclusions**

The analysis of the plasma-induced radical species often involves EPR spectroscopy and the spin trapping technique. This work was aimed at investigating the fate of the products of spin trapping (organic nitroxides) in aqueous solutions exposed to different plasmas. The observed non-zero order decay of 4-oxo-TEMPO, a model nitroxide, was shown to occur non-selectively *via* both the reduction of the nitroxide group and possible ring-opening degradation reactions. By employing various scavengers (glycine, HQ and BQ), the mechanism of nitroxide decay was investigated. With oxygen-free plasmas, decay occurred partially *via* the reduction of the nitroxide group by plasma-generated •H atoms.

The decay of the radical adducts formed in reactions with nitrones was studied using PBN spin trap. The use of isotopically labelled water (D2O) vapour in the plasma feed gas allowed monitoring of the PBN-D spin adduct decay in H2O solution treated by plasmas. It was shown that the spin adduct decayed similarly to the model nitroxide 4-oxo-TEMPO under the same conditions. The excess of the spin trap (unreacted nitrone) acts as a scavenger for the species causing decay and thus enhance the stability of the adduct.

The selectivity of nitroxide decay was further studied for the PTIO/PTI spin trapping system used to detect •NO radical in plasma-exposed media. PTI, a product of the •NO trapping by PTIO, decayed at higher rates than PTIO, and to an even greater extent in the presence of unreacted PTIO. Thus, the use of PTIO in many cases may be inconclusive: the absence of PTI signal does not indicate it wasn’t formed. This complicates selective •NO detection in solutions exposed to plasma.

All these factors make the task of the detection of free radicals in the liquid highly challenging. Stability of spin traps and the spin adducts upon plasma exposure need to be taken into account for accurate analysis of the obtained results.

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