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Feature

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# Analysis of Short-Lived Reactive Species in Plasma−Air−Water Systems: The Dos and the Do Nots

**This Feature addresses the analysis of the reactive species generated by nonthermal atmospheric pressure plasmas, which are widely employed in industrial and biomedical research, as well as first clinical applications. We summarize the progress in detection of plasma-generated short-lived reactive oxygen and nitrogen species in aqueous solutions, discuss the potential and limitations of various analytical methods in plasma−liquid systems, and provide an outlook on the possible future research goals in development of short-lived reactive species analysis methods for a general nonspecialist audience.**

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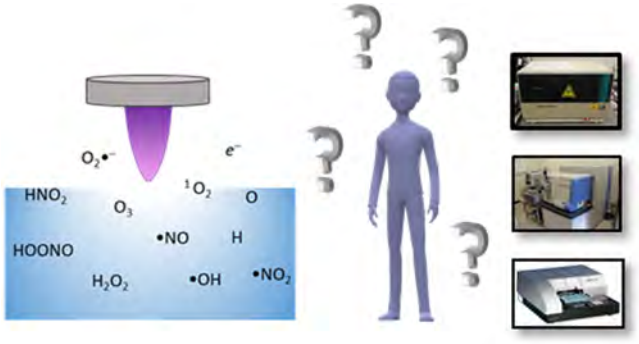


## COLD ATMOSPHERIC PRESSURE PLASMAS: RESEARCH AND APPLICATIONS, REACTIVE SPECIES, AND INTERACTION WITH LIQUIDS

Plasma is an ionized gas, often (controversially[1]) referred to as the fourth state of matter. It contains neutral gas molecules and atoms but also electrons and various types of ions, excited species, and radicals. Plasmas are generally divided into several types based on the operating temperature and pressure. First, we can distinguish (i) high-temperature plasmas, operating at temperatures of (several) million K (as used for fusion research, mimicking the conditions of the Sun) and (ii) low temperature plasmas. The latter can still be subdivided into thermal plasmas, operating at temperatures of a few thousand K (such as inductively coupled plasma used for ICPMS and ICP-OES in analytical chemistry), and nonthermal plasmas, where the gas remains at room temperature (and up to 1000 K) but the electrons are heated to 10 000−30 000 K (1−3 eV). These nonthermal ("cold") plasmas are also called "gas discharges"[2] and operate at either low pressure (e.g., glow discharges used for GDMS and GD-OES in analytical chemistry) or atmospheric pressure (e.g., plasma jets and dielectric barrier discharges, also used as ion sources in analytical chemistry for ambient ionization mass spectrometry).[3,4]

These cold atmospheric pressure plasmas (CAPs) are not only important in analytical chemistry, but they have gained significant interest in recent years due to their unique properties. They can be generated by applying an electric field to a gaseous medium at ambient pressure and near-ambient ("low" compared to thermal plasmas) temperature.[5,6]

CAPs produce various reactive oxygen and nitrogen species (RONS) which define their potential applications[5,7] (Figure 1).

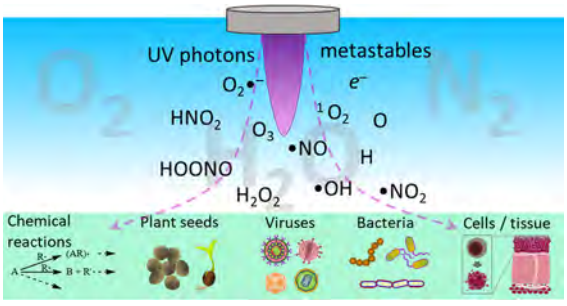


**Figure 1.** Cold plasma in contact with liquids produces various RONS for diverse chemical, agricultural, and medical applications.

The applications include photoresist removal,[8] plasma catalysis,[9,10] thin film deposition and modification,[5,11] production of enhanced nanomaterials,[12] plasma-assisted polymerization,[5,13] wastewater treatment,[11,14] removal of volatile organic chemicals from air,[10,15] conversion of $CO_2$ into value-added chemicals,[16] etc. Moreover, cold plasma is a valuable tool in chemical synthesis.[17,18] Being able to efficiently initiate radical chain reactions in systems comprised of gaseous plasma and liquids,[18] CAP is an attractive alternative to conventional radical initiators.

However, the most burgeoning field of CAP research is biomedical and agricultural applications. The agricultural utilizations of CAPs are enhancement of seed germination, antibacterial and antifungal treatment of plants,[19] similar to food processing, which makes use of the decontaminating properties of cold plasma.[20] The vast area of CAP applications in biomedicine spreads from dentistry (teeth whitening, disinfection)[21,22] and production of surfaces with antibacterial resistance[23] to various sterilization processes,[24,25] wound healing,[24]

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deactivation of bacteria and viruses,[26,27] but the field that recently attracts the most attention is cancer treatment.[24,28−30] In many cases, biomedical CAPs are plasma jets in which plasma is created with a noble gas (Ar[30] or He[27,28]), although air is also used.[31]

The reactive species are created either within the plasma[32] (e.g., using He with $O_2$ admixture[28]) or upon its interaction with the surrounding atmosphere[30,33] ($N_2$, $O_2$, and $H_2O$ vapor in air). The resulting RONS "cocktail" consists of various atomic, radical, ionic, and molecular species, such as O, $O_3$, $^1O_2$, $^\bullet OH$, $O_2^{\bullet-}/^\bullet OOH$, $^\bullet NO$, $ONOO^-$ and $OONOO^-$, $H_2O_2$, $NO_2^-$, $NO_3^-$. These species, when interacting with biological substrates, are responsible for the plasma-elicited effects.[7,24,30] Their analysis, both qualitative ("Is this species present in a particular plasma system?") and quantitative ("At what concentrations?"), is paramount for understanding and tailoring the desired effects of CAP.[34,35]

The gas phase plasma does not interact with dry biological substrates: water plays an essential role in food processing, agricultural and, especially, biological milieu. The primary RONS, such as $^\bullet OH$, $^\bullet NO$, $H_2O_2$, O, $O_3$, are mainly created in the gas phase plasma[7,32,33] and undergo transformations in the liquid, although in certain cases (i.e., plasma discharge to the liquid surface) they can be created in the interface layer of aqueous solutions.[30,35] Secondary RONS, such as, e.g., $^\bullet OH$ and $^\bullet NO_2$ from HOONO, $HNO_2$, and $HNO_3$ from $^\bullet NO$ and $^\bullet NO_2$, are the result of degradation or interaction of the primary RONS with each other or molecules in media.[7,36]

Chemical modeling together with various analytical techniques (e.g., optical spectroscopy and mass spectrometry methods) aids in studying the composition of the gas phase plasma.[7,32,37] Recently, efforts in computational chemistry have addressed the interaction of gas phase RONS with aqueous solutions.[37−39] However, experimental monitoring of RONS in liquid is crucial for model benchmarking and provides the most direct information on the reactive species present in the liquid.

This Feature focuses on the short-lived reactive species induced by CAP in aqueous solutions and methods of their detection, identification, and quantification. The information on the identified limitations of the methods is also important for the generalist audience dealing with the detection of short-lived RONS in solutions, in systems different from CAPs.

## WHICH RONS? TWO TYPES OF PLASMA APPLICATION MODE

The species potentially present in the analyte solutions depend on the actual CAP application mode. One option is a pretreatment of a relevant liquid medium by CAP, with further application to a biological target.[24,27,28,30] In this so-called indirect treatment, the effects of plasma are attributed to long-lived molecular and ionic chemical species, which remain in solution after CAP treatment, such as $H_2O_2$, $NO_2^-$, $NO_3^-$[30,40] (Figure 2). Spectrophotometry is most commonly employed to detect and quantify these species.[41] For example, $H_2O_2$ is often measured using colorimetry with titanium(IV) oxalate or vanadate solutions.[27,30] The colorimetric detection of the nitrate/nitrite pair uses Griess reagent and is not influenced by other long-lived plasma RONS.[42] Many of these detection methods are described in the recent paper by Massima Mouele et al.,[41] albeit without selectivity discussion.

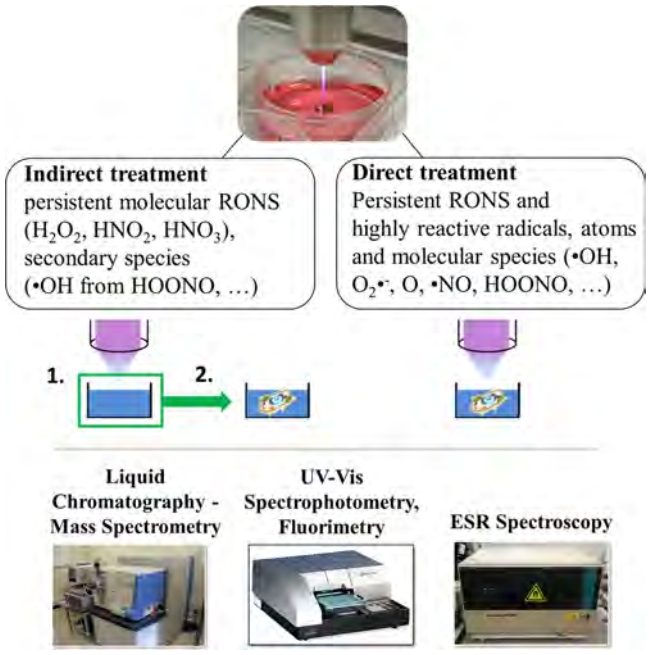


**Figure 2.** Two methods of plasma application: RONS in solutions and main analytical techniques used for their detection.

The second application mode involves direct CAP treatment of the biological target (e.g., bacterial or cancer cells in a liquid medium).[24,27] Here, the RONS mixture does not only contain persistent RONS but also the short-lived radical and atomic species: O, $^\bullet NO$, $^\bullet OH$, $O_2^{\bullet-}/^\bullet OOH$ as well as nonradical chemical compounds, such as singlet oxygen $^1O_2$. These species have diverse effects, from direct oxidative stress[7] to regulation of various cellular processes.[43]

Importantly, they were shown to play a critical role in bacterial inactivation,[44] cancer cell apoptosis,[45] and immunogenic cell death[46] and were suggested to be the major effectors in the degradation of organic compounds in water.[47] Therefore, their analysis in liquid in contact with plasma is of immense importance for the deconvolution of plasma chemistry and effects.[35]

Similar to the long-lived RONS, the short-lived ones are also often measured using spectrophotometric methods.[41,48] However, colorimetric and fluorometric systems consisting of complex organic molecules, and based on either decay or induction of color or fluorescence, lack selectivity (see detailed discussion in Analysis of Short-Lived RONS).

Other reported analytical methods include liquid chromatography coupled with mass spectrometry (LC−MS) of the RONS-modified chemical substrates and electron spin resonance (ESR) spectroscopy.[27,32,33,44]

In plasma-liquid systems, the selectivity of analysis becomes detrimental: plasma generates many different RONS, which are delivered to the liquid at the same time. The other problem arises from the analysis being only semiquantitative in most cases. In the following section, we discuss the main three analytical methods used for the analysis of plasma−air−water systems in terms of these problems.

## ANALYSIS OF SHORT-LIVED RONS

**Optical Spectroscopic Analysis of Plasma-Induced RONS.** Optical spectroscopic methods are probably the most widely used methods for the detection of short-lived RONS via modification of substrates, both historically and based on the



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equipment availability. Some of them used in plasma-liquid research are described in the recent review by Massima Mouele et al.[41]

Many of the chemical reactions used in these methods were adapted by the plasma community from known analyses in biological milieu and are sometimes used disregarding the limitations. Here, we present a general description of the methods with several examples to show some selectivity-based limitations.

Spectroscopic methods are comprised of UV−vis spectrophotometry and fluorimetry (measurements of the intensity of induced or reduced color or induced fluorescence upon reactions of chemical probes with the plasma-produced RONS in liquids) and direct UV spectrophotometry. The latter technique was used to detect $ONOO^-$ based on its characteristic absorption, although high-pH medium is required to stabilize $ONOO^-$ for subsequent UV analysis.[49]

Colored dyes are degraded by CAP-induced RONS, and the loss of color can be quantified using UV−vis spectrophotometry.[50] Some of the most commonly used dyes used to assess ROS are methylene blue and methylene red,[47,51] which are often used in research of CAP for removal of pollutants during wastewater treatment.

The main disadvantage of this method is the nonspecific degradation of such dyes. For instance, decoloration of methylene blue can be a result of reactions with $^{\bullet}OH$, O, and $O_3$.[52] $O_2^{\bullet-}$ can also be detected by degradation of dyes[53] with similar selectivity issues. For example, the induction of chemiluminescence ascribed to the presence of $^{\bullet}OH$ and $O_2^{\bullet-}$ (i.e., nonselectively) was observed at the CAP-liquid interface when alkaline solutions of luminol were exposed to plasma.[54]

Similarly, induction of fluorescent properties of chemical molecules is also used.[55,56] Terephthalic acid (TA) is very often used to detect $^{\bullet}OH$ in CAP-liquid systems.[57,58] The method is based on the induced fluorescence due to the formation of hydroxy-substituted TA. However, possible degradation of TA by plasma species (e.g., combination of $O_3$, $^{\bullet}OH$, $H_2O_2$ and UV photons[59]) is usually not taken into account.

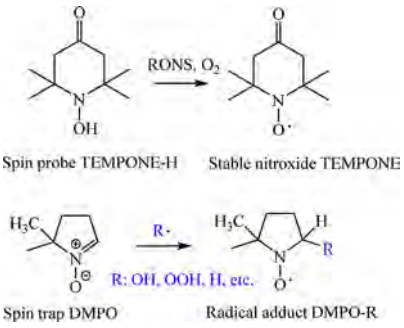
Thus, optical spectroscopy methods can be used for both qualitative and semiquantitative, albeit in most cases very nonselective, detection of the plasma-induced RONS in liquids. The advantage of the method is the possible direct visualization requiring no equipment for a qualitative analysis a simple "present/not present" and "less/more" assessment can be done by the naked eye.[54] An interesting and important use of colorimetry and fluorimetry is introducing respective chemical probes in aqueous compartments of a gel to investigate the depth of the RONS penetration into the gel as a tissue model.[60]

**ESR Analysis.** ESR is the most direct method of radical detection in liquid systems. It is used for the detection of paramagnetic compounds in general and free radicals (systems with unpaired electrons) specifically.[61]

In most cases, free radicals are too short-lived to be detected directly. A technique of "trapping" radicals by reacting them with organic molecules, usually nitrones, is therefore used.[62] Spin traps undergo addition reactions with radicals, yielding more persistent radical adducts, which can be monitored by ESR within reasonable time scales (minutes to hours).[41,63]

Another method is using a spin probe: usually a hydroxylamine of a stable nitroxide.[64] Upon reaction with RONS, a stable nitroxide is formed via the abstraction of an H atom from the hydroxylamine group, and the concentration of this nitroxide radical is measured. Both methods are illustrated in Scheme 1.

**Scheme 1. Formation of ESR-Detectable Nitroxide Radicals from the Spin Probe 1-Hydroxy-2,2,6,6-tetramethyl-4-oxo-piperidine (TEMPONE-H) and the Spin Trap 5,5-Dimethyl-1-pyrroline *N*-Oxide (DMPO)**




These two methods of radical detection are frequently used in biological milieu, both in liquid media and intracellularly.[65] They have been adopted by the plasma community in the past decade and since then widely employed in cold plasma-liquid systems, where they are used to detect $^{\bullet}OH$[66−68] and $O_2^{\bullet-}$,[66,68] $NO^{\bullet}$,[69,70] O and $O_3$,[27,33,71] and $^1O_2$.[27,66] Extensive ESR detection studies covering many of the biologically relevant CAP-induced RONS in aqueous solutions were performed.[70,72] We have previously used spin trapping of the radicals produced from isotopically labeled water to investigate the sources (vapor or liquid) of RONS induced in treated solutions.[32,33,73] Spin trapping was also used to monitor radicals produced from organic solvents in a polymeric solution treated by CAP[12] and in water−alcohol mixtures.[74]

However, there are both general and specific limitations associated with ESR detection of radicals via spin trapping which are sometimes ignored, or otherwise unknown, in the community. We will try to address them here.

(1) First, the detection of radicals by ESR (not limited to plasma-liquid systems) is seldom quantitative. The concentrations of the radical adducts or nitroxides from hydroxylamines are easily obtained using calibration with solutions of stable radicals (e.g., 2,2,6,6-tetramethylpiperidine (TEMPO) or similar stable nitroxides) of known concentration.[32,33,70] However, these values represent only a fraction of the total amount of radicals entering the liquid, which surprisingly is sometimes overlooked.[67] Free radicals undergo various reactions: recombination ($^{\bullet}OH$ to $H_2O_2$), side reactions with water molecules ($^{\bullet}NO$, $^{\bullet}NO_2$), reactions with organic components of the media and other scavengers,[18,27,70] etc. This limits the amount of the trapped radicals based on specific reaction rates. Nevertheless, the changes of these trapped amounts correspond to the changes of the total concentration of radicals (or atoms),[33,71] so qualitative trends can be revealed and yield useful information.

(2) The second limitation is the nonselectivity of spin probes and spin traps. However, these two types of ESR "reagents" require rather different selectivity-related considerations, as we demonstrate with several examples.

In a recent study, TEMPONE-H (see Scheme 1) was used to detect $O_2^{\bullet-}$, $^{\bullet}NO$, and peroxynitrite $ONOO^-$(short-lived at physiological pH),[75,76] due to its high affinity toward these species. However, TEMPONE-H also reacts with $^{\bullet}OH$.[77] Moreover, hydroxylamines of stable nitroxides are easily oxidized, even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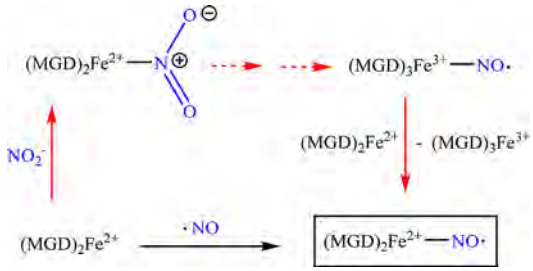
by $O_2$ from air[69,78] and thus also by $O_3$, O, and other ROS produced by plasma. This, once again, is a general difference between biological systems (where only, e.g., $ONOO^-$ and $O_2^{\bullet-}$ are produced by biological bodies in vitro[56,79]) and plasma–air–liquid systems, where a much larger variety of RONS is delivered into the liquid at the same time.

The nonselectivity of spin trapping, on the other hand, is a useful property rather than a disadvantage, because many radicals can be identified simultaneously from the same spectrum. For example, spin trap 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) forms relatively stable radical adducts with $^{\bullet}OH$ and H.[33] In a mixture of radical adducts, each adduct has a unique spectral pattern, due to different hyperfine values defined by the interactions of an unpaired electron with nearby nuclei. In other words, DMPO–OH is distinguishable from, e.g., DMPO-H due to different hyperfine values, using ESR simulations.[32,33]

In certain cases, however, this nonselectivity can produce false-positive results and needs to be taken into account when performing spin trapping experiments. One of the important cases is spin trapping of the CAP-produced $^{\bullet}NO$. In CAP-liquid systems, delivery of both $^{\bullet}NO$ radicals and $HNO_2$ to the liquid is feasible. A spin trap comprising a complex of *N*-methyl-D-glucamine dithiocarbamate (MGD) with iron(II) ion forms $(MGD)_2Fe^{2+}$-NO radical adducts, both with $^{\bullet}NO$ and $NO_2^-$ (Scheme 2).[80]

**Scheme 2. Formation of the NO Adduct in Reactions of *N*-Methyl-D-glucamine Dithiocarbamate (MGD)–Iron(II) Complex with Nitric Oxide and Nitrite Anion**[a]




[a]Side reactions leading to the adduct are marked with red arrows.

Similarly, DMPO–OH can be formed as a result of a nucleophilic attack of $H_2O$ molecules on a carbon atom of the C=N bond in DMPO.[81,82] In these cases, control experiments with no radical production by plasma[69] are required to evaluate the contribution of the side reactions leading to false-positive results (i.e., not from direct spin trapping).

Another example is the oxidation of 2,2,6,6-tetramethylpiperidine (TEMP) or other cyclic amines to the respective stable nitroxides. Directly adapted from biological systems,[83] this reaction has been used to detect $^1O_2$ in water exposed to CAP.[66,70,72] However, unlike those systems, cold plasma produces other RONS, which can oxidize TEMP. Takamatsu et al. used $NaN_3$ as a specific quencher for $^1O_2$ to evaluate its contribution to the formation of the nitroxide radical.[72] Note however that while this method is suitable for media with physiological pH, $NaN_3$ also reacts with $O_3$ under basic conditions.[84] In our previous work we showed that besides $^1O_2$, the only other plasma-induced RONS that can oxidize TEMP are $O_3$ and O.[33] By comparing the densities of $O_3$ and O in the gas plasma with the trends of TEMPO formation in the liquid, the main contributor to this oxidation can be determined.[71] This, however, would be specific to each particular plasma-liquid system.

It is worth mentioning that the (non)selectivity feature can sometimes be an artifact of a spin adduct degradation. For example, the stability of DMPO-OOH radical adduct is low, and the adduct readily transforms into DMPO–OH.[85,86] In such cases, using a scavenger to eliminate one of the possible contributors to the final adduct formation may be required. As an example, superoxide dismutase can be used to estimate the contributions of $^{\bullet}OH$ and $O_2^{\bullet-}$ on the detected DMPO–OH.[66]

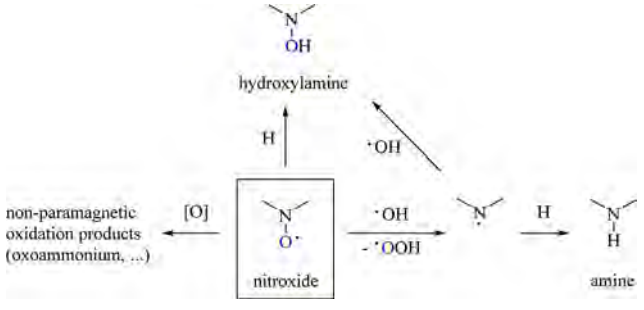
This brings us to the next limitation, inherent to ESR in liquids treated by CAPs.

(3) The third limitation is the degradation of nitroxides in plasma–liquid systems. Both spin traps and spin adducts are organic molecules and as such are prone to degradation by plasma-induced RONS. It was suggested that organic pollutants (and model dye molecules) are degraded in plasma-treated liquids by $^{\bullet}OH$ radicals, O or $O_3$, and solvated electrons from plasma.[14,47,87,88] Similar pathways are possible for spin traps and adducts. Indeed, the spin trap 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline *N*-oxide (DEPMPO) was shown to degrade and form a detectable carbon-centered radical in aqueous solutions upon plasma treatment.[33] *N*-*tert*-butyl-$\alpha$-phenylnitrone (PBN) can undergo degradation to *tert*-butyl hydronitroxide upon reactions with $^{\bullet}OH$.[89] DMPO is nonselectively oxidized to 5,5-dimethyl-2-pyrrolidone *N*-oxyl (DMPOX) by, e.g., $^1O_2$[90] or media-derived oxidants such as hypochlorite.[91,92] In many cases, this does not present a problem due to a large excess of unreacted spin trap. However, in plasma–liquid systems, and especially in systems when plasma is ignited without gas flow,[46,93] mass transfer within the liquid may be limited.[35] This can result in only the top layer of solution reacting with RONS. In this case, the degradation of the spin trap and/or spin adduct may dominate, resulting in a decrease of the obtained ESR signal. It is therefore important to study the development of the adduct concentration within the experimental time frame.[32,33,71]

Spin adducts are only relatively stable, and undergo decay over extended periods of time.[61,65] Thus, standardization of experimental procedures (i.e., time between plasma exposure and ESR measurements) is necessary to obtain any reliable information.

Regarding the CAP–water systems, spin adducts also react with various CAP-induced radicals as well as O and/or $O_3$, yielding nonparamagnetic compounds (Scheme 3). This was demonstrated in plasma-treated water for stable nitroxide TEMPO and a PBN–$^2$H radical adducts, which decayed with similar rates, suggesting similar decay pathways.[69] The reactions of the nitroxide group with $^{\bullet}OH$, H, $O_3$/O, and other

**Scheme 3. Loss of Nitroxide Moiety via Reactions with Plasma-Induced ROS**



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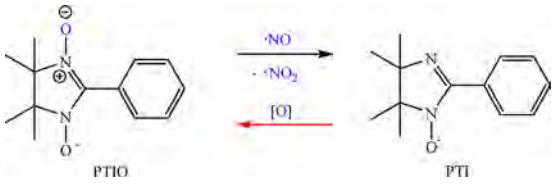
plasma-induced RONS may lead to complete loss of the ESR signal and thus affect even the semiquantitative results.[69,94,95]

Using an excess of the spin trap to avoid the loss of the signal upon decay becomes impractical when the spin trap itself is a radical: the ESR signal of the product will be dwarfed by the signal of the spin trap. This can be demonstrated with 2-phenyl-4,4,5,5-tetramethylimidazoline 1-oxyl-3-oxide (PTIO) spin trap or its derivatives, which are used to detect •NO in CAP-exposed solutions.[56,72] PTIO reacts with •NO to produce 2-phenyl-4,4,5,5-tetramethylimidazoline 1-oxyl (PTI)[96] as shown in Scheme 5. Since both PTIO and PTI are nitroxides, they lose their radical nature in CAP-treated water due to reactions with plasma-induced RONS (see Scheme 3). Moreover, PTI can be oxidized back to PTIO by plasma-induced ROS (Scheme 4). As a result, the decay rate of the product (PTI)

**Scheme 4. Formation of Imino Nitroxide PTI via Reaction of Nitronyl Nitroxide PTIO with •NO[a]**




[a]A reverse reaction is marked with a red arrow.

may even exceed that of the reagent (PTIO).[69] This can lead to a false-negative result in •NO detection.

Despite its limitations, ESR analysis is the most demonstrative tool in analyzing the mixture of (radical) RONS produced by cold plasma in liquids. Moreover, a useful technique of spin trapping can be used in combination with other techniques, as we show below.

**Liquid Chromatography and Mass Spectrometry of Chemical Substrates Modified by RONS: Less Direct, Less Selective.** The spin trapping technique discussed in the previous section can be used in combination with (tandem) mass spectrometry. The adducts of DMPO, DEPMPO, and other nitrone spin traps can be analyzed not by EPR but instead using liquid chromatography–mass spectrometry (LC–MS).[97,98] Certainly, this technique needs to be used with caution, as nitroxides may decay during the analysis, e.g., during electrospray ionization.[99]

In general, mass spectrometry is a versatile technique that can be used for the analysis of stable products, formed in reactions of chemicals with plasma-induced RONS. However, just like with any other method, the complexity of plasma–liquid systems arises from the large variety of RONS in the gas phase plasma, which creates selectivity concerns.

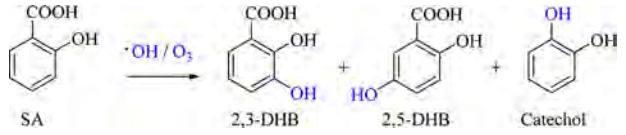
Amino acids, such as, e.g., cysteine, are viable "fingerprint" probes for the detection of RONS in solutions exposed to CAP. Depending on the dominant RONS present in different plasmas, cysteine undergoes different transformations, which can be tracked using the MS analysis of the products.[100,101] The presence of $ONOO^-$ and $ClO^-$ (a highly oxidizing and bioeffective species formed upon plasma exposure of chloride-containing solutions[44,91]) in plasma-treated media can also be assessed using LC–MS. The detection is based on the mass spectrometric analysis of the modified L-tyrosine in aqueous media after plasma exposure. Here, one of the formed products, 3-nitrotyrosine, is ascribed to originate from $ONOO^-$ and •NO radicals formed by CAP.[102,103] However, the nitration of tyrosine is highly nonselective and can occur in reactions not only with $ONOO^-$ and •NO but also $•NO_2$, $NO_2^-$, etc.[104] This limits the applicability of the method to a general presence of RNS in CAP–liquid systems rather than specific species.

Aromatic substitution reactions of phenol enable detection of O as well as •NO, $•NO_2$, and •OH radicals. Nitration and nitrosation of phenols with subsequent high-performance liquid chromatography (HPLC) analysis of the products can be used to detect $ONOO^-$ in aqueous solutions after exposure to CAP. Although several species can lead to the formation of the same product (e.g., nitrosophenol is formed with both •NO and $ONOO^-$; nitrophenol with $•NO_2$, $HNO_2$, and $HNO_3$), the dominating reactions are highly pH dependent.[36] Similarly, HPLC analysis of the hydroxylated products of phenol can be used to detect •OH in liquids in contact with plasma.[42] However, the selectivity is once again an issue: this hydroxylation can also occur with CAP-produced solvated O atoms.[105,106]

HPLC can be employed without MS analysis. This requires calibration of the HPLC equipment with the analyzed compounds. Salycilic acid (SA) is used in CAP–water systems for the detection of plasma-produced •OH. HPLC (even without MS) can be used to detect the hydroxylation product of SA: 2,5-dihydroxybenzoic acid (2,5-DHBA).[107] It was also shown that reactions of SA with •OH in CAP-treated aqueous media lead not only to 2,5-DHBA but also 2,3-DHBA and catechol.[108] HPLC analysis thus requires initial calibration of the specific method (eluent, column, flow, etc.) to estimate the retention time of the products and thus obtain quantitative results on their concentrations. However, it has not been taken into account that the hydroxylated product formation is likely nonselective: it can be caused by $O_3$[109] and possibly O atoms (similar to reactions of phenols; see above).

Thus, the quantitative measurements require assessment of all reaction products and are largely influenced by the presence of other plasma-induced RONS (Scheme 5).

**Scheme 5. Reaction of Salicylic Acid (SA) with •OH and $O_3$**




Aqueous solutions of dimethylsulfoxide were employed in the detection of •OH as well. The products of the reaction, formaldehyde (HCHO) and methanesulfinic acid, are analyzed, e.g., by HPLC (HCHO is first subjected to derivatization with 2,4-dinitrophenyl hydrazine).[57,110,111] However, the results may not be quantitative due to the possible degradation of the analyzed product: HCHO is over-oxidized to $CO_2$ by •OH in the presence of $O_2$.[111]

Thus, chromatography and/or mass spectrometry present valuable analytical options for the analysis of plasma-produced RONS in liquids, albeit not always quantitatively straightforward. Chemical modification of substrates may be nonselective and require complex optimization of analytical conditions together with careful kinetic considerations.

## SUMMARY AND PERSPECTIVES

In this Feature, we discussed the possibilities of the main analytical methods used for the detection of short-lived RONS induced by CAP in liquids. Many of these methods are directly



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Table 1. Analytical Techniques Used for the Detection of Short-Lived RONS Created in Plasma−Air−Water Systems

| Analytical Technique | Plasma RONS Detected | Advantages | Limitations |
|---|---|---|---|
| UV−vis spectrophotometry, fluorimetry | •OH, $O_2^{\bullet-}$, O, $O_3$, $ONOO^-$ | Analyzed compounds are stable. Presence of many RONS can be visualized. Nondestructive. | Indirect. Highly nonselective degradation of dyes. Nonselective induction of chemiluminescence or fluorescence. |
| HPLC, LC-MS(/MS) | •OH, $O_2^{\bullet-}$/•OOH, O, $O_3$, •NO, $•NO_2$, $ONOO^-$ | Analyzed compounds are (often) stable. | Indirect. Nonselectivity of the reactions of chemical probes with plasma-induced RONS. |
| ESR spectroscopy | •OH, $O_2^{\bullet-}$/•OOH, O, $O_3$, $^1O_2$, H, •NO, $ONOO^-$ | The most direct method. More selective than others. Many radicals can be identified simultaneously from the same spectrum. Nondestructive. | Nonselective formation of nitroxides from hydroxylamine spin probes. Nonselective formation of spin trap adducts. Oxidative degradation of spin traps by plasma RONS. Decay of spin adducts by plasma RONS: inherent, nitroxide reactions, oxidation of PTI back to PTIO. |

adapted from biological and chemical systems. In these environments, the analytical techniques are based on spin trapping or substrate ("probe") modification with subsequent LC, MS, ESR, or UV−vis analyses.

The main difference between cold plasma−air−water systems and traditional biological and chemical systems is that in CAP, all (or many) types of RONS are present at the same time. This results in nonselective formation of products (e.g., hydroxylation of phenol), nonselective induction/decay of color or fluorescence (methylene blue reactions with •OH and $O_3$), enhanced degradation of spin traps/spin adducts, etc. This is summarized in Table 1.

We also discuss the limitations of the methods in general, applicable in a wide range of systems with short-lived RONS. Some of the limitations of, e.g., spin trapping are universal and should be taken into account during analysis.

Besides the short-lived RONS discussed above, some other may be present in plasma-treated liquids and their detection and quantification is required. For example, peroxynitrite ($ONOO^-$) is a "sought-after" chemical compound in CAP−water systems,[112] but peroxynitrate ($OONOO^-$) is rarely mentioned, although it can be responsible for most of the bactericidal action of plasma in liquids.[113] Solvated electrons delivered by CAP can initiate radical reactions,[18,114] but their detection in liquids so far has been limited to spectroscopy with a laser diode array[115] and pH measurements in a chlor-alkali process.[116] Like peroxynitric acid[117] or other RONS, free electrons are induced in the media only by some types of CAP: this depends on the configuration of the CAP system.[5,32,33,35]

This means that *not all plasmas are created equal*: the "cocktail" of reactive species produced in a dielectric barrier discharge (DBD) can be different from that created by an Ar plasma jet. For example, $^1O_2$ is created only by some plasma jets[72] but not by others.[33] This is not to mention various configurations of the jets or DBDs.[35] Accordingly, the analytical methods should be selected as best suited not only for specific reactive species but also for the specific CAP−air−water system. The limitations of the analysis methods are general, but in some cases RONS that can interfere with the analytical procedures are simply not present.

Careful choice of analytical methods, optimization of analytical procedures, and comprehensive control experiments can provide an expanded view of the plasma−air−water systems. The complete understanding of such systems, using the expertise from chemistry, biology, and physics, is required for their further development and tailored applications in chemistry, agriculture, food industry, and medicine.

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### Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

### Notes

The authors declare no competing financial interest.



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