Synergetic effects of atmospheric plasma and UVA treatment for eye infection pathogens disinfection

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***Abstract*—We report a combined method of using atmospheric surface micro-discharge (SMD) with UVA exposure to provide a fast and efficient method against eye infection pathogens. 4 kinds of typical eye infection bacteria were chosen and treated under SMD treatment only, UVA exposure only and 5min SMD treatment followed by 1 or 2 min UVA exposure in buffered solution. The combination of plasma treatment and UVA exposure presented the best antibacterial effect, especially for the UV-resistant bacteria strain, Serratia marcescens. This synergetic method has been found to have no selectivity on bacterium type. The possible mechanism of the synergetic effects was preliminarily proved to be caused mainly by photodecomposition of nitrite. UVA photolysis analysis of pure nitrite, H_2O_2 solution and their mixture indicated the formation of peroxynitrite in buffered solution partly contributed to the anti-bacterial effect in this work.**

Keywords—surface microdischarge; UVA; synergetic effects; anti-bacteria; UV resistant

I. INTRODUCTION

In recent years, atmospheric pressure plasma for biomedical application have attracted huge attention for its great potential on cancer treatment, dental disinfections, wound healing, stem cell differentiation, medical equipment sterilization, etc[1-7]. Both in vitro and in vivo tests have been conducted, even on human trials. At the same time, various plasma devices have been created for different uses according to the requirement of clinic[8-9]. Treatment protocols are modified step by step to reach the best results, from single plasma treatment to multiple treatments, from direct plasma treatment to treat with clinic pre-preparation, from sole plasma treatment to combine with other effective drugs or treatment devices. Atmospheric pressure low temperature plasma could rapidly inactivate bacteria, spores, fungi, or virus, even in the form of biofilm both on surfaces and aqueous solutions. Lots of researching groups are trying to find out the mechanisms of plasma interacting with the microorganism or living tissues, however, they are complicated from case to case and have not been well understood yet. Generally speaking, the anti-microbial effect of atmospheric pressure low temperature plasma is mainly brought by the charged particles, highly reactive species (O_3, NO_x, H_2O_2, $OH^{\cdot}$, O, O_2^{-}, etc), electrical field, or photons created by plasmas. In the case of air plasmas acts at the gas-liquid interface, $OH^{\cdot}$, $NO^{\cdot}$, $NO_2^{\cdot}$ and peroxynitrite have been proved to be the strong anti-microbial scavengers in plasma activated water (PAW) at low pH[10-12]. However, for treatment of soft material or tissue like contact lenses, direct plasma treatment may affect its function through etching or deposition. Besides, strong acidic/alkaline environment is not suitable as well. It has been reported that surface micro-discharge (SMD) working in NO_x mode with Ultraviolet (A) (UVA) treatment had strong anti-bacteria ability in neutral solution (pH around 7)[13].

In this work, the synergetic antibacterial effects of 4 standard ISO ATCC strains of eye infection were investigated by using an atmospheric SMD and UVA-LED emitting. The mechanism of the synergetic effect of SMD and UVA was analyzed by measuring the kinetic dynamics of photolysis of both pure and mixed solution (nitrite and H_2O_2) before and after UVA radiation as a function of time.

II. MATERIALS AND METHODS

A. Experimental Setup

Fig.1 (a) shows the configuration of the experimental setup for plasma treatment. A typical SMD device driven by a Neon sign power supply (Vmax=2.5kV, Frequency=25kHZ) was used to create atmospheric plasma in all the experiments. The copper electrode was connected to the AC power, a glass plate was used as the dielectric sheet, and the stainless steel mesh was grounded. When proper voltage was applied, the plasma would be generated in the space between the mesh and the glass plate. This device was operated in 'indirect mode', in which the plasma would not directly contact the sample during treatment. A high voltage probe (P6015A, Tektronix,) was used to detect the output voltage and the voltage waveform was monitored by an oscilloscope (TDS 2024C, Tektronix,).

A glass vial with either phosphate-buffered saline (PBS; PH 7.4; Sigma) or 150 μL bacteria suspension in PBS was placed at the bottom center of the discharging chamber. The height of the glass vial was 4 cm. Distance between the top of the glass vial and the mesh was fixed at 5 mm. After plasma treatment, the solution was well mixed for 5 s with a cap and immediately sent for Ultroviolet-A LED (Nichia, NC4U133A(T)) exposure, seen in Fig.1 (b).

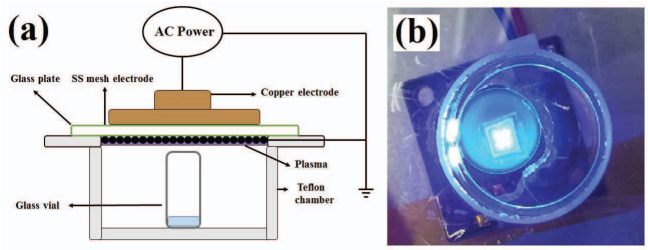

Fig.1 Experimental setup.

This work was supported by the National Natural Science Foundation of China (51907076).

979-8-3503-4667-1/23/$31.00 ©2023 IEEE

2023 IEEE 6th International Electrical and Energy Conference (CIEEC) | 979-8-3503-4667-1/23/$31.00 ©2023 IEEE | DOI: 10.1109/CIEEC58067.2023.10165859

B. Gas-phase and aqueous-phase chemistry

Gas-phase products was measured by a Fourier-transform infrared spectroscopy (FTIR) (FTS 3000, DIGILAB) to show the active species generated by plasma in the discharging chamber. IR-transparent (ZnSe polished disc, International Crystal Laboratories) windows were situated 2 cm below the powered electrode on the chamber wall (made of acrylic glass) for FTIR measurements. Signals were averaged for 50 times (takes about 30 s) for each point. The absorption FTIR spectra at time point of 30 s, 1min, 2 min, 3 min, 4 min and 5min were sampled. We defined the plasma ignited time as time 0 s.

Nitrite concentration in aqueous phase as a function of time was measured by using Griess reagent (SIGMA-ALORICH). In all cases, 100 µL solution of each sample or diluted sample was transferred into a hole of 96 well plate and 100 µL Griess reagent was immediately added into the hole for reaction. Blank group was made by 100 µL PBS and 100 µL Griess reagent. Samples were kept in dark for at least 30 min. Then, a UV-Vis spectrometer (SPECTRA max M2, Molecular Devices) was used to measure the absorption value at 548 nm.

Titanium sulfate colorimetric method was used to measure H_2O_2 concentration in aqueous solution. 100 µL sample and 50µL titanium sulfate reagent were added into a hole of 96 well plate, pre-add 10 µL sodium azide solution into the sample if nitrite is expected to exist. After that, immediately measure the absorption at 407 nm using a UV-Vis spectrometer. For all the test, at least 3 samples were repeated for each treatment point.

C. Antimicrobial Experiments

4 types of eye infection pathogenic bacteria were chosen for test in the experiments: Pseudomonas putida (ATCC 12633), Serratia marcescens (ATCC 13880), Staphylococcus epidermidis (ATCC 35984), Staphylococcus warneri (ATCC 27836). Cells were grown in LB (lysogeny broth, Fisher Scientific) medium to OD_{600} 1.0, and diluted with PBS to an initial concentration around 10^6 ~10^7 cfu/mL before treatment. The antimicrobial experiments were divided into 5 groups: 1) The control group; 2) UVA exposure only; 3) 5 min plasma treatment only; 4) and 5) 5 min plasma treatment followed by 1 or 2 min UVA exposure. As for group 4) and 5), firstly, add 150 µL bacteria suspension into the glass vial, and treated by plasma for 5 min; then, cap the glass vial, mix the solution and the active species in the air gap for 5 s. Next, remove the cap, and put the glass vial above the UVA-LED lamp for exposure. After these treatments, the bacteria suspension was diluted in PBS and plated on LB agar. Colonies were counted after 72 h for calculating the reduction log (N_0/N), where N_0 is the original number of viable cells in control group for each kind of bacteria, and N is the viable number present in a treated sample.

III. Results

A. Active species in gas phase and NO_2^- concentration in PBS

Fig.2 shows the FTIR spectrum of the species created by the SMD plasma device *vs.* time. The gas phase reactive species under this condition consist of N_2O, HNO_3, NO_2, no O_3 presents. The absorbance of each species increases with the discharging time. As reported by *M. P*, 3 kinds of modes could appear under different applied power region, in low power density region, O_3 is the dominate species, which is called O_3 mode; in high power density region, appears NO_x mode (nitrogenous compounds); and between these two, the transition mode[14][15]. Apparently, in our discharging condition, the SMD device was operated in NO_x mode.

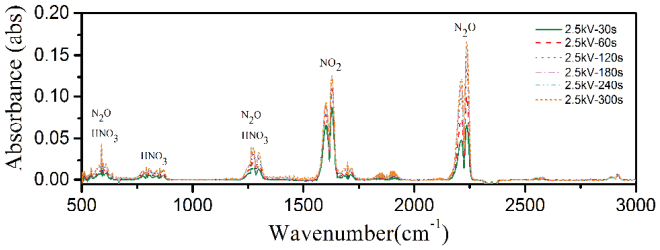

Fig.2 Typical FTIR spectrum.

Fig.3 shows the NO_2^- concentration *vs.* time in aqueous phase. It can be seen that the NO_2^- concentration in PBS after plasma treatment continues to rise and exceeds ~1.25 mM at 5 min. The measured concentration of H_2O_2 after 5 min SMD treatment is around 80 µM.

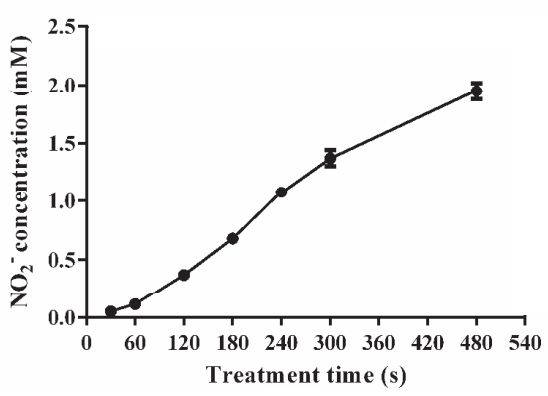

Fig.3 NO_2^- concentration in PBS right after plasma treatment.

Fig.4 shows the aqueous concentration of NO_2^- and H_2O_2 after UVA exposure as a function of time. The concentrations of both pure solvent (pure NO_2^- or H_2O_2 solution) and mixed solvents (NO_2^- with H_2O_2) were measured after UVA exposure. The prepared solvent concentrations were used other than plasma treated solution for a constant initial concentration, and H_2O_2 concentration was increased to show a better visible results. As can be seen from the plot, in the case of pure NO_2^- solution and NO_2^- with H_2O_2 solution, NO_2^- concentration shows obviously decrease after UVA treatment. 1 min and 2 min UVA exposure only decomposes about 10%~20% of NO_2^-. 12 min of UVA exposure decreases the NO_2^- concentration (initial concentration 1 mM) down to 60%~70% of the initial concentration. In the mixed solution, the NO_2^- decomposes slower than that of in the pure NO_2^- solution.

Pure H_2O_2 solution shows much slower kinetic rate constant k around $-1.5*10^{-4}s^{-1}$ according to the simple equation $\ln\{[H_2O_2]t/[H_2O_2]0\} = kt$. When NO_2^- exists, H_2O_2 shows great chemical reaction under the UVA exposure, almost half of H_2O_2 was decomposed after 12 min UVA exposure. Although the concentration of H_2O_2 used in this measurements is much higher than that created by the SMD device after 5 min, however, it could give us the expected results as a reference. The possible mechanism will be discussed in the discussion section.

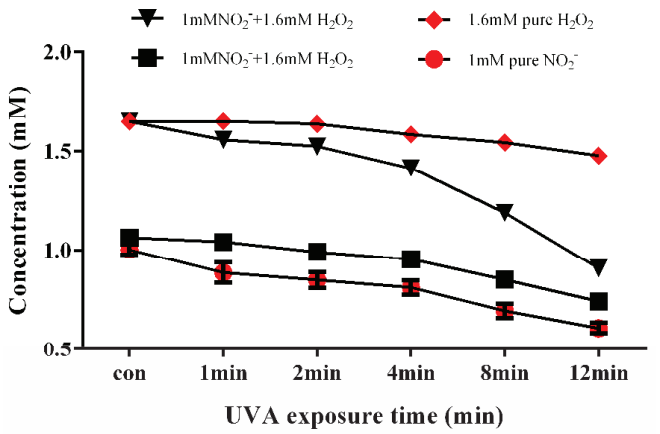

Fig.4 Chemical dynamics of UVA photolysis of nitrite and H_2O_2 in pure/mixed solution. Black triangle: H_2O_2 concentration in mixture solution; Black square: Nitrite concentration in mixture solution; Red rhombus: H_2O_2 concentration in pure H_2O_2 solution; Red circle: Nitrite concentration in pure nitrite solution.

B. Anti-bacteria effect

The anti-bacteria effect of only UVA treatment, only plasma treatment and synergistic anti-bacteria effect of plasma and UVA exposure of all 4 kinds of eye infection pathogens was all tested. Fig.5 and Fig.6 are the anti-bacteria results of 4 different kinds of pathogens (Pseudomonas putida, Serratia marcescens, Staphylococcus epidermidis, Staphylococcus warneri) involved in eye infection under different treatment condition. Fig. 5 presents the log reduction results of the anti-bacterial effect by UVA exposure only. Columns in Fig.6 show the anti-bacteria effect of 5 min plasma treatment only, 1 min UVA exposure, 5 min plasma treatment plus 1 min UVA exposure, 2 min UVA exposure and 5 min plasma treatment plus 2 min UVA exposure, respectively. Each data represents the mean log reduction of at least 3 trials, and error bars represent the standard deviation about the mean value.

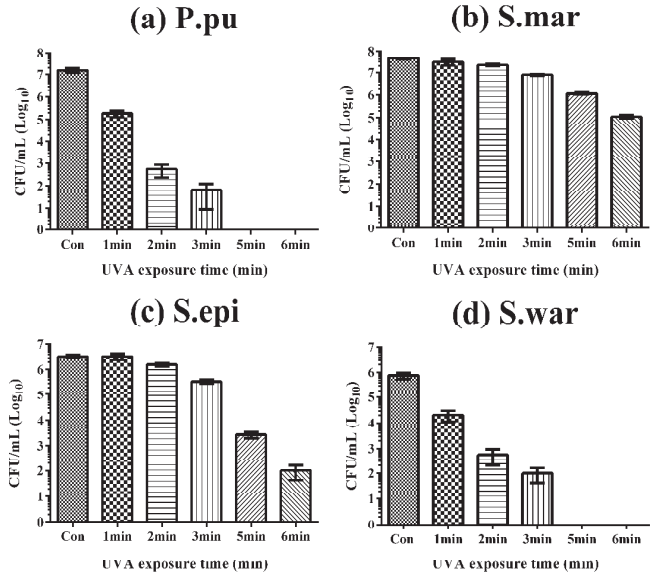

Fig.4 The anti-bacteria effect of 4 different kinds of pathogens by UVA exposure only *vs.* time.

Among these bacterium, Pseudomonas putida and Serratia marcescens belong to the Gram-negative bacteria, the rest Gram-positive. It is important to point out that Serratia marcescens (S.mar) is a species of rod-shaped gram-negative bacteria in the family Enterobacteriaceae, and also a kind of UV-resistant bacteria[16]. For all other kinds of bacteria except S. mar, 4.5~7 log reduction were observed after 6 min UVA exposure. However, for Serratia marcescens, 5 min UVA exposure only can achieve 1 log reduction, and less than 2 log reduction with 6 min exposure.

Unlike UVA exposure, it seems that the anti-bacteria effect of SMD in buffered solution has no significant relationship with the bacteria types. When 5 min plasma treatment followed by only 1 min to 2 min of UVA exposure, additional 2~4.5 log reduction was obtained comparing to the UVA exposure only condition in all the treated samples. Comparing 2 min UVA exposure treatment to S. mar with the 5 min plasma treatment plus 2 min UVA exposure, there is ~ 4.5 log difference between these two treatment methods.

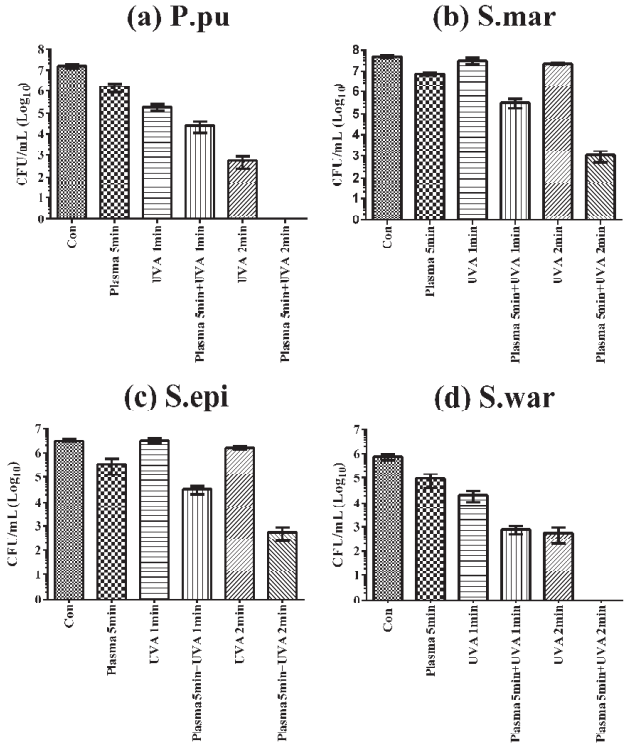

Fig.5 The synergetic anti-bacteria effect of 4 different kinds of pathogens by plasma and UVA.

IV. Discussion

In this investigation, we used an atmospheric pressure low temperature plasma device, surface micro-discharge device (SMD), combined with UVA (365 nm) radiation to provide a novel and promising method for eye infection pathogens disinfection. SMD can create various kinds of anti-microbial active species, such as RNS (NO, NO_2, HNO_2, N_2O_4, HNO_3, etc) and ROS (O_3, O*, OH, H_2O_2, ect). These active species have been proved to be strong scavenge against bacteria, fungus, and virus in different existing forms[17].

The four kinds of bacteria used in this experiments are all involved with eye infection. The vast majority of bacteria corneal infections are related to contact lens wear and sustained by Pseudomonas aeruginosa, with potential for corneal perforation and even blindness without treatment[18]. P. pu is usually used as a nonpathogenic alternative bioremediation agents for P. aeruginosa. In some case, it could be the main sustain isolate for conjunctiva infection[19], and would present drug resistance.

Staphylococcus is the most frequent isolates from bacteria conjunctivitis[20]. S. epi and S. war are both belonged to the Staphylococcus family. S. epi is the most common isolate for ocular infection among other opportunistic pathogens[21], and is known as resistant to methicillin[22]. It is also the most common pathogen causing acute postoperative endophthalmitis by forming biofilm on the intraocular lenses[23]. S. mar is a rod-shaped gram-negative bacterium in the family of Enterobacteriaceae, serves as a source of nosocomial infections, which is relatively resistant to standard sterilization and disinfection protocols[24]. It is also a common pathogen of eye infection and a frequent cause of keratitis[25]. Resistance to β-lactams, cephalosporins, and aminoglycosides have been reported by many researches[26], and it is recognized as a UV-resistant bacteria by producing the red pigment called prodigiosin which offers protection against excessive UV in sunlight, serves as an antibiotic, and has cytotoxic qualities[27].

As can been seen from Fig. 5 and Fig. 6, for all kinds of bacteria, 5 min plasma treatment only results 0.5~1 log reduction against bacteria in buffered solution, which is similar to the results in [13] and shows no bacteria selectivity. However, for the results of only UVA treatment, S. mar presents strong UV resistance comparing to other bacteria followed by S. epi, which shows 0.2 log reduction after 2 min and 1.5 log reduction after 5 min. And for all the 5 min plasma treatment with 2 min UVA exposure group, additional 3~4.5 log reduction were obtained. Unlike drugs and UV exposure, the synergetic effect of plasma with UVA exposure shows no bacteria selectivity against all the bacteria strains tested in our experiments, which provides a more efficient and broader germicidal way against microorganisms.

Gas phase and aqueous phase of air plasma chemistry have been vastly studied in recent years[28-30]. The reactions depends on different types of discharge, its energy, gas components, and very importantly the pH value of contacting liquids[21-23]. Different plasma devices have their own feature and even for the same device when operated in different conditions, the anti-microorganism mechanism would be different[14]. PH environment is a key feature for anti-microorganism effects[34]. Acidic pH value caused by air plasma discharging in water contributes a lot of the antibacterial properties of plasma-activated water[35]. However, when plasma interacts with human body which needs to avoid strong acidic/alkaline environment or for some pH-sensitive material, using buffered solution for investigation is highly necessary. In this study, we used SMD device operating in NO_x mode combining with UVA exposure to treat inactive different type of eye infection pathogens in buffered solution (PBS, pH=7.4). The complex mechanism of the synergetic effects hasn't been well understood yet. In [13], a hypothesis on the synergetic effect by plasma and UVA radiation was proposed. The main chemical reactions under this discharging conditions of active species generating are listed as follows:

$$e + O_2 \rightarrow O + O + e \quad (1)$$

$$O + O_2 + M \rightarrow O_3 + M \quad (2)$$

$$N_2 + O \rightarrow NO + N \quad (3)$$

$$O_3 + NO \rightarrow NO_2 + O_2 \quad (4)$$

$$O + NO + M \rightarrow NO_2 + M \quad (5)$$

$$NO_2 + NO_2 + H_2O \rightarrow NO_2^- + NO_3^- + 2H^+ \quad (6)$$

H_2O_2 formation in gas phase through the recombination reaction of OH radicals generated by the reactions between electron (e) and water molecular in the air.

$$OH\cdot + OH\cdot \rightarrow H_2O_2 \quad (7)$$

The possible reaction of active species acting with UVA (photons) in PBS (PH=7.4) are listed below:

When exposing to UV radiation, nitrite will react with the photon through a well-known reaction:[36][37]

$$NO_2^- + h\nu + H_2O \rightarrow NO + OH^- + OH\cdot \quad (8)$$

While

$$H_2O_2 \xrightarrow{h\nu} OH\cdot + OH\cdot \quad (9)$$

Under neutral environment,

$$NO + H_2O_2 + OH\cdot \rightarrow O{=}NOOH + H_2O \quad (10)$$

$$O{=}NOOH \leftrightarrow OH\cdot + NO_2\cdot \quad (11)$$

According to the reported literature, in plasma activated buffered solution (PABS), the concentration of NO_2^-, NO_3^- and H_2O_2 will stay constant even after a week, while under acidic environment, the reactive species will go through series reactions[11][31]. It is obvious that the decomposition of NO_2^- and H_2O_2 in Fig. 4 is due to the involvement of photon provided by UVA (365 nm) exposure. The measured concentration of H_2O_2 after 5 min of SMD exposure is around 80 μM. As mentioned in results section, the calculated reaction rate constant of equation (9) is around $-1.5*10^{-4}$ s^{-1}, which is similar to the reported values[38]. When NO_2^- exists, the decomposition rate of H_2O_2 has been largely increased, which shows a strong evidence to support the reaction of equation (10). Moreover, when no H_2O_2 exists in the solution, the decomposition rate of NO_2^- is faster compared to that with H_2O_2. This could be explained as follows: without H_2O_2, NO_2^- is decomposed into NO by photon radiation through reaction [8]; NO plays as strong anti-bacterial scavenger in this process under neutral environment other than acidic nitrite. It is short lived and hard to dissolve in water which might escape into air. However, when H_2O_2 exists, NO reacts with H_2O_2 and form peroxynitrite equation (10), which captures NO from escaping. The forming peroxynitrite would decompose into $NO_2\cdot$ through reaction equation (11). And then reforms NO_2^- in solution, making the decomposing rate slower than that in pure NO_2^- solution. The peroxynitrite and $OH\cdot$ produced by UVA photolysis in the bacterial suspension also partly contributed to the bacterial inactivation process. All of these results would support the hypothesis on the synergetic effect by plasma and UVA radiation proposed in [13]. More work needs to be done for this complex process in the future.

V. CONCLUSION

The synergetic effects of atmospheric plasma and UVA radiation for eye infection pathogens disinfection were investigated in this study. The combination of SMD treatment operating in NO_x mode with UVA (365 nm) exposure shows remarkable increased anti-bacteria effect (another 3~4.5 log reduction comparing to UVA exposure) against 4 kinds of testing bacteria involved in eye infection. Comparing to the log reduction results of only plasma treatment or only UVA exposure, this synergetic method shows no selectivity on bacteria type, such as gram positive or negative, UVA resistant, drug resistant, etc. The mechanism of the synergic effect of this atmospheric pressure plasma and UVA radiation is proved to be the photodecomposition of nitrite by UVA exposure, and peroxynitrite formation through reaction between NO and H_2O_2 in solution.

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