Cold Plasma Generation of Peracetic Acid for Antimicrobial Applications

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ABSTRACT: This study compares how a helium plasma jet activates peracetic acid (PAA) from tetraacetylenediamine (TAED) and acetic acid (AA). Hydrogen peroxide (H_2O_2) generated from the plasma jets reacts with TAED resulting in the formation of PAA which further dissociates into AA. The by-product AA can also react with H_2O_2 to form PAA, which might also be useful for antimicrobial applications when coupled with plasma. Equivalent concentrations of TAED and AA solutions are used to compare the formation of PAA after activation with a helium plasma jet. Our results showed that the concentrations of both H_2O_2 and PAA in plasma-activated TAED (PAT) are higher than plasma-activated AA (PAAA), and that PAT is more efficient in reducing the growth of *Pseudomonas aeruginosa* and *Staphylococcus aureus*; the pathogens commonly found in wounds. The results are attributed to the presence of more acetyl donor groups in TAED, resulting in the formation of higher concentrations of PAA and H_2O_2 .

I. INTRODUCTION

Plasma-activated water (PAW) has been shown to be an effective antibacterial solution against a range of bacteria for medical and food related decontamination applications.^{1–3} Even though the chemistry of PAW can be varied in a number of ways, its main antimicrobial effects are usually mainly attributed to the combination of hydrogen peroxide (H_2O_2) and nitrogen oxide species that reduce the pH of water.^{4–7} The reason for this is that most plasma treatments produce a much higher concentration of H_2O_2 compared to other generated reactive species, and they also produce peroxyntous acid that decreases the water's pH. Acidification is known to enhance the antibacterial property of H_2O_2 .^{1–5}

The use of PAW as an antibacterial agent has been investigated across several industries, including medicine,^{2,8,9} agriculture,^{10,11} and food.³ PAW has been shown to have a high level of antibacterial efficacy in the above applications, including against antibiotic-resistant bacteria.^{12–14} These results have raised enthusiasm that PAW can potentially offer an antibiotic-free solution to combat the rise of antimicrobial resistance (AMR) in healthcare and in our environment. However, given that the use of PAW as an antimicrobial agent is still relatively new and for many applications is still at the research phase and has not seen widespread industry use, it is still unknown how the

longer-term exposure of PAW to microorganisms might also result in facilitating AMR. Early work suggests that bacteria are indeed able to develop resistance against plasma treatments.¹⁵ The main hypothesis in these studies is that bacteria release catalase in response to oxidative stress produced by the plasma treatment.¹⁶ Catalase neutralizes H_2O_2 , which can prevent oxidative damage to the host bacterial cell.

To overcome the bacteria's oxidative defense mechanisms, we have developed a new PAW formulation by adding the antimicrobial precursor acetyl donor molecules tetraacetyl-ethylenediamine (TAED) and pentaacetate glucose (PAG)¹⁷ into the water (prior to plasma treatment). Plasma-generated H_2O_2 activates the acetyl donor molecules, generating a potent oxidant, peracetic acid (PAA), that acts in synergy with H_2O_2 and other reactive oxygen and nitrogen species (RONS) generated by plasma in the water [Reaction (1) in Fig. 1].¹⁸ Also, Fig. 1 shows the acetyl donor groups CH_3CO on TAED and on the by-product AA of the TAED- H_2O_2 reaction. PAW in combination with plasma-activated acetyl donors was shown to be superior in the decontamination of Gram-negative and Gram-positive bacteria as well as the SARS-CoV-2 virus compared to the use of PAW alone.¹⁷

This present study aims to provide further insights into the chemistry of the plasma-activated acetyl donor reaction to better understand the importance of the processing parameters in PAA generation. Specific focus is given to the plasma activation of TAED compared to acetic acid (AA). As shown in Fig. 1, Reactions (2) and (3), AA is a by-product produced by the reaction of an acetyl donor with H_2O_2 . Because AA also contains an acetyl group, it can subsequently react with H_2O_2 to form PAA through a reversible reaction [Fig. 1, Reaction (2)]. Therefore, by carrying out this study we can determine if other agents such as AA can be used instead of commonly used acetyl donors such as TAED, as well as provide new information on the chemical pathways involved in the enhancement of the antibacterial activity of PAW with PAA.

II. METHODS AND MATERIALS

A. Plasma Jet Setup

Figure 2 is a schematic of the experimental setup with a photograph of the plasma jet. The plasma jet assembly consists of a 150-mm-long, 3-mm-inner-diameter glass tube tapered to

FIG. 1: Formation of PAA through the reaction of TAED or AA with plasma-produced H_2O_2

FIG. 2: (a) Schematic of experimental setup; (b) He plasma jet treating an acetyl donor solution

600 μm at the nozzle. A single 15-mm-long external ring copper electrode is wound onto the glass tube 50 mm above the nozzle. The flow rate of helium (He) through the glass tube is fixed at 1 standard liter per minute (SLPM). Plasma is generated by supplying a high voltage of 10 kV p-p (peak-peak) at 25 kHz to the copper electrode using a commercial power supply (PVM500, Information Unlimited, USA). Voltage and current waveforms are measured using a high-voltage probe (Pingtek Electronics, Taipei, Taiwan) and a current monitor (Pearson Electronics, Palo Alto, CA, USA), respectively, with the waveforms recorded on an oscilloscope (Sigilent Technologies, Solon, OH, USA). The optical emission signals of the discharge are measured with a spectrometer (Ocean Optics HR4000 CG-UV-NIR).

B. Preparation and Analysis of Antimicrobial Solutions

Antimicrobial solutions used to compare the formation of PAA with the plasma jet consisted of (1) 5 mM TAED (CAS No. 10543-57-4, Sigma Aldrich, St. Louis, MO, USA), (2) 5 mM AA (CAS No. 64-19-7, Sigma Aldrich), and (3) deionized water (DIW; resistivity 18.2 M Ωcm). A 400- μL volume of TAED, AA, or DIW was transferred to a well of a 96-well plate (Corning Inc., Corning, NY, USA) and treated with the plasma jet for 20 min. The distance between the plasma jet nozzle and the top of the DIW surface was 3 mm. Analysis of the characteristics of the antimicrobial solutions was performed using the three techniques described next.

1. Measurement of H_2O_2 Concentration

Measurement of H_2O_2 concentration was carried out using a reaction to assess the horseradish peroxidase (HRP; CAS No. 9003-99-0, SigmaAldrich) catalyzed oxidation of o-phenylenediamine (OPD CAS No. 95-54-5, SigmaAldrich) as previously described.¹⁷ HRP catalyzes the oxidation of OPD with H_2O_2 , which forms a final yellow product, 2-3-diaminophenazine with an absorbance maximum at 450 nm. A tablet of OPD was dissolved in 10 mL DIW, and 20 μL of 2-mg/mL HRP was added to the solution. 20 μL

antimicrobial solution (or H_2O_2 ; CAS No. 7722-84-1, Sigma Aldrich) was added to the wells of a 96-well plate containing 180 μL dissolved OPD-HRP, and incubated for 15 min at ambient temperature in the dark. The absorbance at $\lambda = 450$ nm of the solutions was measured on a microplate reader (Synergy LX, Biotek). The absorbance measurements directly correlated with the H_2O_2 concentration, which was subsequently calculated from a line-of-best-fit calibration curve (Fig. A1 in Appendix A).

2. Measurement of PAA

The PAA concentration in the plasma-activated solutions was measured using the following reagents: (1) potassium iodide (KI; CAS No. 7681-11-0, Sigma Aldrich), (b) catalase (CAS No. 9001-05-02, Sigma Aldrich), and (3) PAA (Fisher Scientific, Hampton, NH, USA). Then 20 μL of 100-mg/mL catalase was mixed with 5 μL known concentrations of PAA (or plasma-activated solutions) and incubated for 15 min. Any H_2O_2 present in the antimicrobial solution was quenched by the catalase, resulting in the oxidation of KI mainly from PAA due to its significantly higher oxidative capacity compared to the remaining plasma-produced oxidative species.¹⁷ Finally, the oxidation of KI was measured by adding 5 μL test solution in 195 μL of 100-mM KI and incubated for 15 min. The absorbance of the resulting solution was measured at 350 nm using a plate reader (Synergy LX, Biotek). The calibration curve constructed with known concentrations of PAA (Fig. A2 in Appendix A) was then used to measure the concentration of PAA formed in PAT or PAAA.

3. H_2O_2 Activation of TAED and AA

This experiment was conducted in order to compare the oxidation efficiency of TAED and AA following reactions with different concentrations of H_2O_2 . To begin, 20 μL of different concentrations of H_2O_2 were added into 180 μL of 100-mM KI and incubated at room temperature for 15 min. Afterwards, the absorbance was read at 350 nm using a plate reader (Biotek, Winooski, VT, USA).

C. Antimicrobial Assays

Common wound pathogens *Pseudomonas aeruginosa* (*P. Aeruginosa*, PA01) and *Staphylococcus aureus* (*S. Aureus*, H560) were used for studying the bactericidal effect of plasma-activated TAED and AA solutions. A 400 μL solution of 5-mM TAED in DIW, 5 mM AA in DIW, and DIW alone were treated with the He plasma jet for 20 min in a 96-well plate (frequency: 20 kHz, He gas flow: 1 LPM, applied voltage: 10 kV p-p). After treatment, 80 μL of the plasma-activated solution was transferred to another well containing 80 μL of the bacteria (initial concentration = 10^6 CFU/mL). The bacterial culture mixed with the plasma-activated solution was incubated for 12 h. The optical density of the solution at $\lambda = 600$ nm (OD_{600}) was used to determine the comparative growth of the bacteria in the various samples.

III. RESULTS AND DISCUSSION

A. Electrical and Optical Characteristics

The electrical and optical characteristics of the He plasma jet used to produce the plasma-activated solutions are shown in Fig. 3. Plasma was generated at 1-SLPM He flow by applying a sinusoidal voltage of 10 kV p-p at a frequency of 25 kHz. Typical current (I) and voltage (V) waveforms of the He plasma jet during treatment of the DIW are shown in Fig. 3(a). Accumulation of wall charges took place on the inner side of the quartz tube during the rising time of the applied voltage. These wall charges gave rise to a current peak. During the falling time, these charges were reversed in the voltage polarity, which gave rise to the current peak in the negative direction.^{4,19,20} The root mean-square (RMS) value of the current was calculated to be 5.91 mA. The I-V waveforms were used to calculate the dissipated power (P) as⁵

$$P = \frac{1}{T} \int_{t=0}^{t=T} I(t) V(t) dt \quad (4)$$

FIG. 3: (a) Typical current and voltage waveforms of the He plasma jet during treatment of DIW; and (b) typical optical emission spectrum of the free stream He plasma jet measured at 3 mm below the plasma jet nozzle

where $I(t)$ and $V(t)$ represent the instantaneous values of current and voltage, respectively, at a time t within a period T . The total dissipated power was calculated to be 0.6 W.

A typical optical emission spectrum of the He plasma jet recorded between 200–900 nm is shown in Fig. 3(b). The spectrum was measured for a free stream plasma jet at 3 mm below the plasma jet nozzle at an integration time of 70 ms. Observed in the spectrum were emissions from the hydroxyl radical ($\cdot\text{OH}$) between 306 and 309 nm, nitrogen second positive system (N_2 SPS) between 296 and 380 nm, nitrogen first negative system (N_2 FNS) between 390 and 440 nm, excited helium between 470 and 707 nm, and atomic oxygen (O) at 777 nm.⁵ Emissions from the $\cdot\text{OH}$ are known to occur due to the dissociation of water vapor molecules present in the feed He gas and ambient air (humidity $\sim 36\%$) while the emissions from excited nitrogen species are due to the dissociation of nitrogen molecules present in ambient air. Emissions from He appeared through electron induced excitation of He from the feed gas and O was formed through the dissociation of oxygen or water vapor molecules. The $\cdot\text{OH}$ observed in the spectrum formed H_2O_2 due to recombination reactions from the $\cdot\text{OH}$ molecules.⁵ H_2O_2 subsequently reacted with TAED and AA, resulting in the formation of PAA as depicted in Fig. 1.

B. Measurement of H_2O_2 Concentration

Measurement of H_2O_2 concentrations in PAW, plasma-activated AA (PAAA) and plasma-activated TAED (PAT) were carried out using the protocol described earlier. The calibration curve used to calculate the H_2O_2 concentration is shown in Fig. A1. The corresponding concentrations of H_2O_2 measured for PAW, PAAA, and PAT after 20 min of plasma treatment are shown in Fig. 4. The H_2O_2 concentration was the highest in PAT (10 mM), followed by PAA (6.5 mM) and then PAW (2.5 mM). The higher concentration of H_2O_2 in PAT and PAAA compared to PAW was due to the dissociation of PAA into H_2O_2 , which is explained in the next section.

C. He Plasma Jet Generation of PAA from the Activation of TAED and AA

H_2O_2 produced by the He plasma jet can react with TAED or AA, resulting in the formation of PAA. This takes place as follows:¹⁸

The intermediate product DAED in Eq. (5) is called diacetylenediamine.

PAA formed in Reaction (5) through the reaction of TAED with H_2O_2 remains in equilibrium with its decomposition product, acetic acid, and 1 molecule of H_2O_2

FIG. 4: H_2O_2 concentrations measured in PAW, PAAA, and PAT after 20 min He plasma jet treatment

[Reaction (6)]. The reversible reaction in (6) shows that H_2O_2 can also react with AA to form PAA. To measure the resultant concentrations of PAA in the plasma-treated solutions, we monitored the ability of PAA to oxidize KI. Catalase was added to the KI solution to neutralize H_2O_2 , which ensured that the majority of KI oxidation was due to PAA and not H_2O_2 or other RONS produced by the He plasma jet.¹⁷ We had previously determined that this method could be used to selectively distinguish the PAA-induced oxidation of KI from other plasma-generated RONS.¹⁷ The concentration of PAA measured in the PAT and PAAA is presented in Fig. 5. The results show that PAT contained approximately two times higher concentration of PAA than PAAA. PAA was

FIG. 5: Concentration of PAA measured for PAAA and PAT after 20 min He plasma jet treatment

not detected in PAW alone as this solution did not contain acetyl donor molecules.¹⁷ The higher concentration of PAA detected in PAT compared to PAAA was due to the multiple acetyl groups in TAED compared to only one group in AA, resulting in double the number of PAA molecules generated per reaction with H_2O_2 .

This explanation was confirmed by following the oxidation of KI by TAED and AA that was activated with different concentrations of H_2O_2 . The results are shown in Fig. 6. Oxidation of KI was similar between the activated TAED and AA at H_2O_2 concentrations ≤ 5 mM. But at concentrations of 10–100 mM, activated TAED produced a higher level of KI oxidation compared to activated AA, and this difference increased as a function of H_2O_2 concentration. These results can be explained by the higher number of acetyl groups in TAED compared to AA as shown in Fig. 1. At lower H_2O_2 concentrations, there were not enough H_2O_2 molecules available to activate all of TAED's acetyl groups, resulting in a level of KI oxidation from PAA similar to that produced by the activated TAED and AA. Whereas at concentrations > 10 mM, the number of available H_2O_2 molecules to activate the extra acetyl groups of TAED (compared to AA) increased, facilitating the production of more PAA molecules that could react with KI.

Because H_2O_2 is neutralized by catalase, an antioxidant released by bacteria as a protective measure against excessive oxidative stress (described in the Introduction), it is possible that PAA in PAAA and PAT can help overcome this form of AMR. To test this, the capacity of PAW, PAAA, and PAT to oxidize KI was tested with and without catalase. Figure 7 shows that the oxidative capacity of PAT is greater than that of PAAA which is greater than that of PAW. In the presence of 100 mg/mL catalase, which can completely neutralize up to 100 mM of the H_2O_2 tested in this study (much more than the maximum H_2O_2 concentration of ~ 10 mM produced in the solutions in this study; see Fig. A3 in Appendix A), it is seen that the oxidative capacity of PAW is nearly eliminated. This result is attributed to the major oxidant in PAW (i.e., H_2O_2) being neutralized by catalase. Even with catalase, however, PAAA is still slightly oxidative. And PAT,

FIG. 6: Oxidation of KI by TAED and AA activated with different concentrations of H_2O_2

FIG. 7: Oxidation of KI by PAW, PAAA, and PAT with and without catalase

with the highest concentration of PAA, is the most efficient at oxidizing KI in the presence of catalase. Therefore, these results show that PAA indeed helps overcome a major antioxidant defense mechanism in bacteria, and consequently potentially helps resist against AMR.

D. Antibacterial Assessment

The antibacterial property of the PAW, PAAA, and PAT solutions was tested by their ability to reduce growth of the common wound pathogenic bacteria *P. aeruginosa* and *S. aureus*. Bacterial growth was assessed by measuring the turbidity of the bacterial broth at OD₆₀₀, where a higher OD₆₀₀ correlates with increased turbidity and consequently more bacteria. The results are presented in Fig. 8. Compared to untreated bacteria, PAW effectively reduced the growth of *P. aeruginosa* and to a lesser degree *S. aureus*. PAAA was more effective than PAW at reducing the growth of both bacteria but still could not completely inhibit the growth of *S. aureus*, presumably due its different Gram-positive membrane providing a greater barrier to the penetration of oxidative molecules compared to the Gram-negative membrane of *P. aeruginosa*. PAT proved the most effective antibacterial agent in this study, achieving complete inhibition of bacterial growth. Therefore, the bacterial assay showed that the number of available acetyl groups from the antimicrobial precursor agent is an important parameter in enhancing the antibacterial efficacy of the plasma-activated solution because it influences the solution's capacity to produce PAA.

IV. CONCLUSIONS

This paper investigated the formation of peracetic acid (PAA) from the plasma-activated acetyl group-containing molecules tetraacetylenediamine (TAED) and acetic acid

FIG. 8: Antibacterial assessment of PAW, PAAA, and PAT compared to untreated bacteria (positive control of bacterial growth)

(AA) for antimicrobial applications. TAED and AA were activated by a helium plasma jet, and the antimicrobial efficacy of the respective solutions was compared to their hydrogen peroxide (H_2O_2) and PAA concentrations and their ability to oxidize a potassium iodide solution. Plasma-activated TAED produced a higher concentration of H_2O_2 and PAA compared to plasma-activated AA, resulting in a greater oxidative capacity of potassium iodide. This correlated with the more potent oxidant plasma-activated TAED exhibiting higher efficacy in killing the common wound pathogens *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The superior antimicrobial efficacy of plasma-activated TAED compared to plasma-activated AA is attributed to TAED having more acetyl groups, which react with H_2O_2 to produce a higher concentration of PAA. Our results suggest that TAED is a better option than AA for antimicrobial applications when coupled with plasma treatments.

ACKNOWLEDGMENTS

The authors would like to thank the EPSRC (Grant Nos. EP/R003556/1 and EP/V00607X/1). E.J.S. acknowledges the Australian Research Council Future (Fellowship No. FT190100263), the National Health Medical Research Council Ideas (Grant No. 2002510), and the Future Industries Accelerator Mobility Scheme MOB024.

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APPENDIX A.

FIG. A1: Calibration curve to deduce concentration of H_2O_2 in PAW, PAAA, and PAT

FIG. A2: Calibration curve to deduce concentration of PAA in PAAA and PAT

FIG. A3: Quenching of different concentrations of H_2O_2 by catalase