

# Aqueous Plasma Pharmacy: Preparation Methods, Chemistry, and Therapeutic Applications

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**ABSTRACT:** Plasma pharmacy is a subset of the broader field of plasma medicine. Although not strictly defined, the term aqueous plasma pharmacy (APP) is used to refer to the generation and distribution of reactive plasma-generated species in an aqueous solution followed by subsequent administration for therapeutic benefits. APP attempts to harness the therapeutic effects of plasma-generated oxidant species within aqueous solution in various applications, such as disinfectant solutions, cell proliferation related to wound healing, and cancer treatment. The subsequent use of plasma-generated solutions in the APP approach facilitates the delivery of reactive plasma species to internal locations within the body. Although significant efforts in the field of plasma medicine have concentrated on employing direct plasma plume exposure to cells or tissues, here we focus specifically on plasma discharge in aqueous solution to render the solution biologically active for subsequent application. Methods of plasma discharge in solution are reviewed, along with aqueous plasma chemistry and the applications for APP. The future of the field also is discussed regarding necessary research efforts that will enable commercialization for clinical deployment.

**KEY WORDS:** plasma pharmacy, aqueous plasma pharmacy, plasma medicine, plasma-activated medium, plasma-stimulated medium, plasma-activated solution

## I. INTRODUCTION

Aqueous plasma pharmacy (APP) falls within the field of plasma medicine, which has been growing rapidly over the past two decades; scientific advancements have revolved around the application of nonthermal plasma plume chemistries for a variety of clinical purposes. In this regard, APP differs from the topical use of plasma plumes onto the surface of body tissues, which has been more extensively studied and which generally includes the direct application of low-temperature (nonthermal or “cold”) atmospheric plasmas, such as jets, plumes, or dielectric barrier discharge systems. Ultimately fundamental plasma physics, life science research, and clinical medical applications all intersect in the APP field. Plasma is defined as an ionized, electronically excited gas containing ions, radicals, and electrons. Nonthermal plasma indicates that the kinetic

energy of the gas atoms, molecules, and ions is lower than the electrons, which results in minimal temperature increase from room temperature.<sup>1</sup> Plasma medicine approaches involve exposing cells or tissues to plasma species to induce destruction (in the case of bacteria and cancer cells) or to harness the therapeutic effects of oxidant plasma species (e.g., to promote angiogenesis and wound healing).<sup>2</sup>

Applications are generally categorized as follows: (1) dermatological applications, such as wound healing and treating scars, ulcers, acne, etc.; (2) selective cancer therapies wherein *in vitro* studies indicate promise toward selective treatment of cancer cells, but this approach is currently limited to easily accessible cancer cells such as melanoma; (3) disinfection and sterilization of planktonic and biofilm bacteria for a variety of surface and topical applications; (4) promotion of angiogenesis, or the growth of new blood vessels; (5) transdermal drug delivery methods, as the plasma plume induces poration and thus increases drug uptake; and (6) bleeding applications, which promotes coagulation during surgical procedures, or for cutting during electrosurgery.

Notably, many universities and institutes internationally are exploring the versatile application of plasma species for therapeutic action with emphasis on both fundamental plasma science and commercial clinical application. Many individual research groups and institutions have recognized the possibilities of plasma applications in health care. Major contributors to the plasma pharmacy field include laboratories at Drexel University, George Washington University, the Leibniz Institute, the Max Planck Institute, Nagoya University, Texas A&M University, and others. In addition to academic research, several companies specialize in plasma tools for use in electrosurgery, such as Bovie Medical Corporation, Olympus, Plasma Surgical, EP Technologies, and US Medical Innovations.

The AJ Drexel Nyheim Plasma Institute at Drexel University includes the Plasma Medicine Laboratory, led by a faculty team with expertise across the fields of biology and medicine as well as electrical, chemical, and mechanical engineering. The Plasma Medicine Laboratory has led the field with seminal publications on dielectric barrier discharge plasma plumes for sterilization, cancer treatment, bacterial inactivation, coagulation, angiogenesis, bone fusion, and a variety of dermal applications including wound, ulcer, and scar treatment.

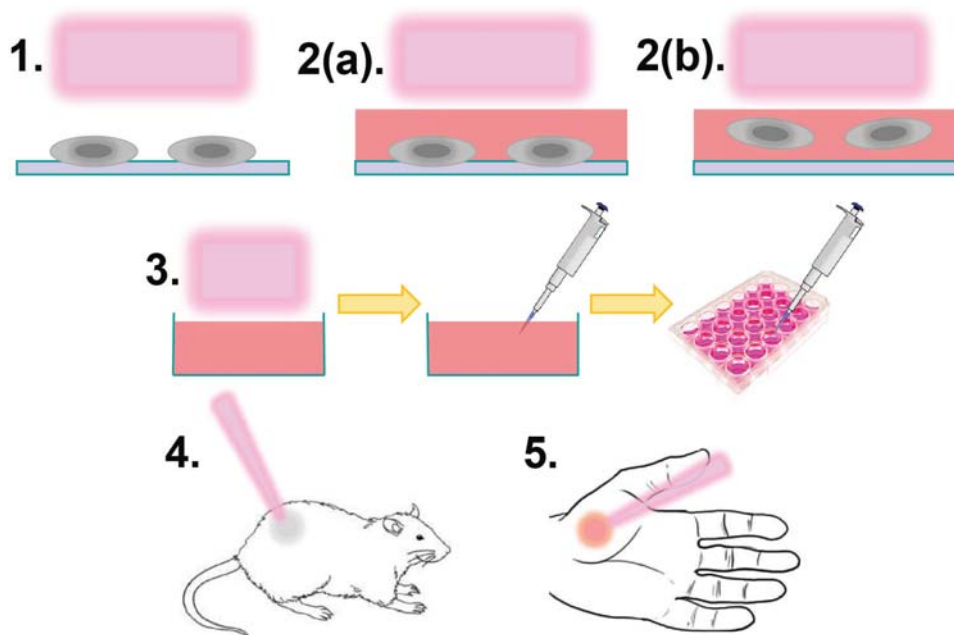
Other translational work has been performed at George Washington University, where Prof. Keidar's team in the Department of Mechanical and Aerospace Engineering has led versatile plasma research in the field of plasma medicine. More specifically, much work has been published outlining the use of plasma plumes for wound healing, cancer treatment, and electrosurgery, as well as specialty plasma manufacturing applications.

Additionally, the Leibniz Institute for Plasma Science and Technology (INP Greifswald e.V.) has been working over the past decade to investigate the application of plasma-plume technology for disinfection, *in vitro* cancer treatment and cell modification, and a variety of topical dermal applications. Notably, the work at Leibniz has resulted in a spin-off company, Neoplas Tools, which specializes in the production and applica-

tion of a proprietary hand-held plasma torch pen, the kINPen; they have three product offerings for dermal applications (e.g. disinfection, wound healing): (1) kINPen® MED for cold tissue-compatible plasma, (2) kINPen® DENT for dental medicine, and (3) kINPen® VET for veterinary applications.

Most of the work completed thus far in the field of plasma medicine falls within the following approaches (Fig. 1): (1) treatment of adhered cells in the absence of media; (2) treatment of cells in the presence of media where the cells are (a) adhered to a substrate or (b) suspended in the media; (3) treatment of media or saline, which is subsequently applied to cells; (4) direct treatment of xenografted tumors in animal models; and (5) direct dermal treatment of animal or human models. Notably, approaches 1–3 include *in vitro* studies, while 4 and 5 encompass *in vivo* work.

Many previous review articles have been published containing in-depth summaries of the plasma medicine field<sup>3–10</sup>; several reviews also specifically cover cancer treatment,<sup>11–19</sup> dermatology,<sup>20,21</sup> and wound healing.<sup>22</sup> Thus, the field of plasma medicine related to plasma-plume applications directly to cells or tissues is becoming well established; however, applications of plasma-activated aqueous solutions have been explored less and represent a more recently emerging field. The focus of this review article is to



**FIG. 1:** Approaches to plasma medicine include (1) direct plasma plume treatment of cells; (2) direct plasma plume treatment of cells in the presence of media where the cells are (a) adhered or (b) suspended in media; (3) plasma treatment of aqueous solution for secondary application to cells; (4) direct plasma treatment of xenografted tumors in animal models; and (5) direct dermal treatment of wounds and other topical ailments.

highlight the recent advances in the plasma modification of aqueous solutions to result in biologically active liquids, which subsequently can be applied internally or externally for a variety of therapeutic applications, referred to as APP. The emerging field of APP is based upon the transfer of plasma species to aqueous solution, which can then be administered secondarily to yield therapeutic effects in cells and tissues.<sup>23</sup>

Herein, we present (a) methods for plasma discharge in aqueous solution, (b) the chemistry of plasma species in solution, (c) biomedical applications for plasma-prepared bioactive liquids, and (d) commercial applications and future directions for the field, all presented within the context of APP. A prior review on plasma pharmacy examined applications in the preparation of bioactive liquids, pharmaceutical preparations, drug transport, and biotechnological processes.<sup>23</sup> However, since its publication in early 2013, much more has been learned about the preparation and application of bioactive liquids via plasma processes, as highlighted below. We also summarize the state of the field regarding methods for plasma discharge and subsequent solution chemistry, where most work has focused on applications in water treatment; however, these findings can be extended to apply to APP as well. Table 1 describes the major players in the field of plasma medicine and the multitude of applications for which plasma systems are being

**TABLE 1:** A summary of the various laboratories and institutes involved in plasma medicine research, predominantly the application of plasma plume technologies for topical (e.g., dermal) applications, and/or to perform *in vitro* cell assay experiments

University / Institute	Application	Ref.
Ajou University	Cancer ( <i>in vitro</i> )	24–30
Changchun University of Science and Technology	Cancer ( <i>in vitro</i> )	31
Clemson University	Melanoma ( <i>in vitro</i> )	32
Conway Institute of Biomolecular and Biomedical Research	Cancer ( <i>in vitro</i> )	33
CSIRO	Cancer ( <i>in vitro</i> )	34,35
Drexel University	Dermal applications; disinfection; melanoma ( <i>in vivo</i> ); cancer ( <i>in vitro</i> )	36–48
Dong-A University	Cancer ( <i>in vitro</i> )	49–52
Eindhoven University of Technology	Disinfection, cancer ( <i>in vitro</i> )	53
George Washington University	Fibroblast modification; wound healing; cancer ( <i>in vitro</i> ); electrosurgery	54–67
Heinrich-Heine University	Skin infection, wound healing	68
Kanazawa University	Wound healing	69
Korea Advanced Institute of Science and Technology	Cancer ( <i>in vitro</i> )	70
Kwangwoon University Plasma Bioscience Research Center	Cancer ( <i>in vitro</i> )	71–73
Leibniz Institute for Plasma Science and Technology	Dermal applications; cancer ( <i>in vitro</i> )	74–88
Max Planck Institute	Dermal applications; cancer ( <i>in vitro</i> )	89–91
McGill University	Cancer ( <i>in vitro</i> )	92,93

**TABLE 1:** (continued)

Nagoya University	Cancer ( <i>in vitro</i> ); anti-fungal; macular degeneration	94–104
National Institute for Laser, Plasma, and Radiation Physics	Cancer ( <i>in vitro</i> )	105
Old Dominion University	Fungal decontamination, cancer ( <i>in vitro</i> )	106,107
Plasma Engineering Research Lab, Texas A&M	Surface decontamination, antibiotic-resistant bacteria, wound treatment, cancer ( <i>in vitro</i> )	108–117
Pohang University	Melanoma	118,119
Pusan National University	Melanoma	120
Queen's University Belfast Centre for Plasma Physics	Nosocomial infection prevention, biofilm eradication, cancer ( <i>in vitro</i> )	121–128
Shahid Beheshti University	Melanoma	129
Universite' d'Orleans	Subcutaneous tumor treatment (murine model)	130–134
University of Buffalo	Melanoma	135,136
University of Campinas	Fibroblast modification	137
University of Iasi	Dermal applications	138
University of Notre Dame	Cancer ( <i>in vitro</i> )	139
York Plasma Institute, University of York	Cancer ( <i>in vitro</i> )	140

applied. Representative publications from each group are shown, and the major applications published by each group are highlighted.

## II. METHODS OF PLASMA DISCHARGE IN SOLUTION

Several approaches to enabling plasma discharge near or directly submerged in an aqueous solution are presented in the literature. Much of this work stems from the application of plasmas for water treatment, where transfer of potent short- and long-lived oxidant species to solution can result in *in situ* treatment. Plasma systems represent an attractive synergistic approach to water treatment over conventional stand-alone advanced oxidation processes (i.e., ozone, UV, hydrogen peroxide) because multiple oxidation mechanisms are employed for *in situ* disinfection and organic compound oxidation.<sup>141–143</sup> As such, a variety of plasma generation systems, including pulsed corona discharge, dielectric barrier discharge, and contact glow discharge electrolysis, have been investigated for water treatment applications.<sup>144</sup> Additional applications for aqueous plasma include environmental, chemical, material functionalization, synthesis, and industrial clean-up applications.<sup>145,146</sup> Comprehensive reviews are available in the literature that describe the fundamental physics of various systems;<sup>147</sup> herein, we provide a condensed review of these methods within the context of APP.

There are many different methods of producing plasma discharge in solution, but they generally can be split into two main categories: (1) surface-liquid discharges, where plasma is discharged in a gas near the surface of the liquid, and (2) direct discharges, where plasma is discharged within the solution. Reviews specific to methods of plasma

generation provide great detail regarding these methods.<sup>141,147</sup> Some of the main methods for producing plasma in each of these categories are outlined below, although there are many possible variations and combinations of these systems. A summary of these methods is shown in Table 2. Any of the methods below are applicable to the production of plasma-activated solutions for use in APP.

## A. Surface-Water Discharges

### 1. *Gliding Arc Discharge*

The gliding arc discharge method features the formation and movement of an arc discharge between two diverging electrodes in the presence of gas flow, illustrated in Fig. 2(a). The flow essentially drags the discharge upward, which causes the arc to extend laterally as it rises, eventually extinguishing as the gap between electrodes becomes too great. The discharge has arc and nonthermal glow properties due to its transient nature. Liquid can be introduced with the feed gas, in which case radicals and other oxidants are produced by the interaction of the arc, feed gas, and liquid.<sup>142</sup> Additionally, it has been shown that  $\text{H}_2\text{O}_2$ ,  $\text{O}_3$ ,  $\text{HNO}_3$ , and other reactive species can be produced by a gliding arc discharge near the surface of an aqueous solution.<sup>148</sup>

This method has been shown to be effective when a spray of liquid is introduced with the feed gas, especially at generating  $\text{H}_2\text{O}_2$  and other volatile species. A possible explanation is that the droplets are much more effective at sequestration of volatiles than a larger body of liquid due to the increased surface tension. The disadvantage of this type of system is that scaling to a larger volume of liquid treated is difficult due to the primarily gas phase nature of the system.

### 2. *Dielectric Barrier Discharge*

In the dielectric barrier discharge (DBD) approach, the plasma is produced in a gas in close proximity to the surface of the liquid flowing through the apparatus (Fig. 2b). In its simplest configuration, DBD is the gas discharge between two electrodes, separated by one or more dielectric layers. The gap between electrodes is usually on the order of millimeters, with a broad range of voltages required for discharge depending on the configuration. The presence of the dielectric barrier inhibits the transition from glow to arc discharge, thus ensuring stable, nonthermal plasma.<sup>149</sup> Reactive species produced in the gas interact with the liquid at the surface region.<sup>142</sup> Researchers have found that these DBD plasmas can produce a variety of reactive species including, for example, hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), ozone ( $\text{O}_3$ ), and nitric acid ( $\text{HNO}_3$ ) in aqueous solutions, which have been shown to have efficacy for applications including wound treatment and sterilization.<sup>148</sup>

**TABLE 2:** A summary of the main plasma discharge methods

Method	Characteristics	Advantages	Disadvantages
Gliding arc discharge	A gas/water mixture flows between two diverging electrodes	Improved sequestration of volatile reactants such as $H_2O_2$ ; continuous flow	Low liquid flow; more complex system; difficult to scale
Dielectric barrier discharge	Plasma discharges from an internal electrode through a porous dielectric barrier and into a gas/water mix	Exposes liquid to reactive species more effectively than point/plane-to-plane approaches; can be continuous flow; some scale-up potential	More complicated apparatus; higher power usage; lower liquid flow
Surface-water point-to-plane	Pointed electrode at high voltage above solution with a grounded plane electrode in solution	Simpler system that does not require as much power as direct discharges; easy to generate plasma; air as an electrode insulator	Plasma generated species only have contact with the surface of the solution; difficult to scale
Direct discharge without feed gas injection	Usually point-to-plane, with a vapor layer (formed at a certain voltage) coating electrode	Less complex system; feed gas or special electrodes not required; plasma-generated species have direct contact with solution	Joule heating required to produce vapor around electrodes for discharge facilitation; heavy electrode wear
Direct discharge with feed gas injection	Usually point-to-plane with special electrodes at high voltage that release gas to facilitate plasma	Bubbles enable better tuning of chemistry via feed gas and enhance diffusion of plasma into solution; Joule heating not required to induce vapor around electrode; can be modified into a continuous flow system	Extensive electrode wear; possible quenching of plasma from liquid



### *3. Point/Multi-Point/Plane-to-Plane Atmospheric Discharge*

In the atmospheric discharge approach, sometimes called a glow discharge, a discharge is initiated between the surface of the liquid and an external electrode (Fig. 2c–d). If the solution contains electrolytes, the discharge current actually flows through the electrolytic solution. In other atmospheric discharge applications, the plasma is produced in general proximity to the surface of the liquid. In both cases, as current flows chemical reactions such as radical production can take place in both the liquid and the gas phase.<sup>141</sup>

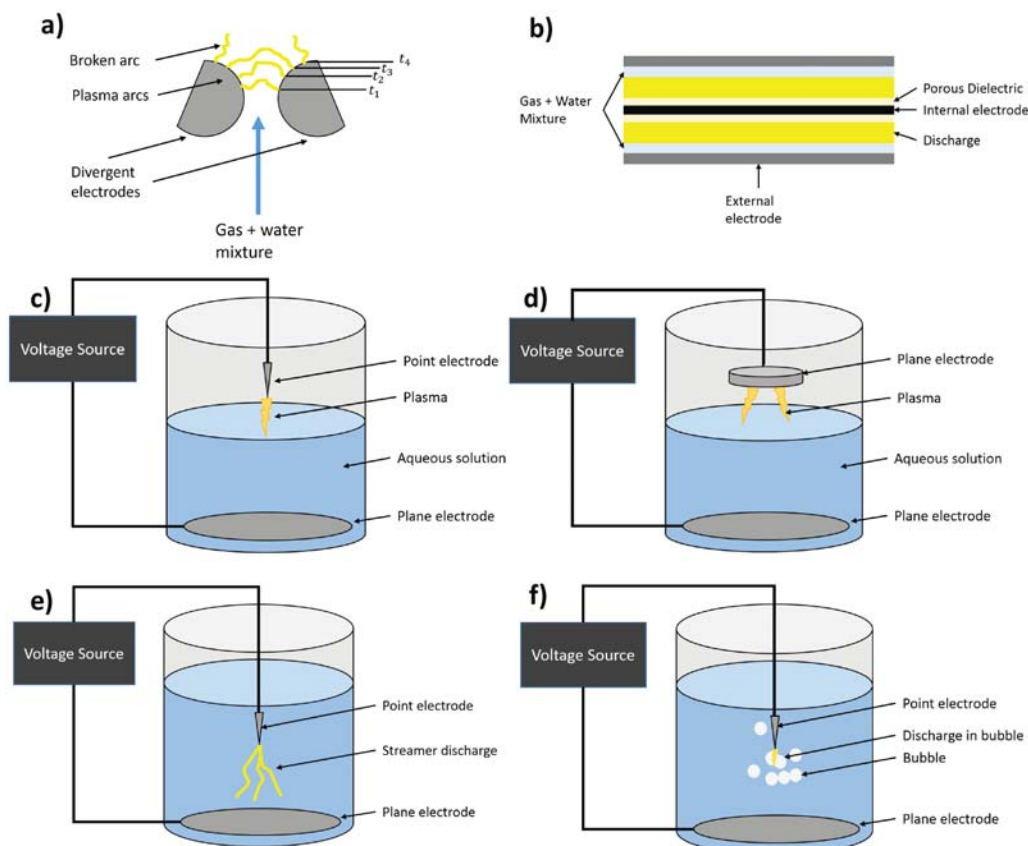
The most common version of this plasma discharge method is the point-to-plane configuration. The advantages of this type of system include ease of electrode replacement, freedom to treat liquids independent of their composition, and ease of plasma generation. This method is effective because the air surrounding the point electrode acts as an insulator, which helps focus the current density to the electrode tip thereby generating plasma more readily. The disadvantage is that this approach creates essentially a surface treatment rather than a volumetric treatment, so to treat an entire liquid volume, the system must rely on diffusion or convection. This presents a problem for scale-up, although it has been proven to be effective at small-scale liquid treatment. More recently, it has been shown that placing the high-voltage electrode in the aqueous phase and the ground in the gas phase above produces larger amounts of hydrogen peroxide in the liquid phase and ozone in the gas phase,<sup>150</sup> although this configuration still is not scalable for commercial use.

## **B. Direct Discharge Methods**

### *1. Point-to-Plane Liquid Discharge*

A method of achieving the direct interaction of plasma with liquid water is to produce the discharge within the liquid itself. This so-called direct injection method typically involves the application of a fast, high-voltage pulse between submerged electrodes.<sup>142</sup> To produce a pulsed discharge in water, it is necessary to have a high-intensity electric field at the tip of the electrode. One of the major problems of the point-to-plane geometry used in these pulsed corona reactors is that the very large electric field at the high-voltage electrode (up to  $10^9$  V/m) necessary to produce the discharge causes extensive wear on the electrode. To help overcome this technical challenge, the most common method of direct discharge is the point-to-plane geometry, shown in Fig. 2(e–f). The concentrated electric field at the pointed electrode drives localized air bubble formation and subsequent electrical breakdown of the fluid between the electrodes, thereby inducing a plasma. This approach is also used with introduction of feed gas injection which facilitates the formation of gas bubbles in which plasma is discharged. The gas bubbles are distributed throughout aqueous solution, enabling higher surface area between the plasma/gas phase and the surrounding liquid, which facilitates transfer of the oxidant species to solution. Feed gas injection parameters include the choice of gas composition





**FIG. 2:** The following plasma discharges are pictorially represented: (a) gliding arc discharge, (b) dielectric barrier discharge, (c) surface-water point-to-plane glow discharge, (d) surface-water plane-to-plane glow discharge, (e) direct discharge without feed gas injection, and (f) direct discharge with feed gas injection.

and gas flow rate, which subsequently impact the chemistry of the plasma produced, in addition to enhancing the diffusion of radicals into solution,<sup>141</sup> which is addressed in the chemistry section of this review. Radicals are formed within the gas bubbles at the water–gas interface, and it has been demonstrated that radical formation increases greatly when gas was bubbled into the discharge region.<sup>151</sup>

A larger-scale solution to this was developed by Manolache et al. in the form of a dense-medium plasma reactor (DMPR), which is a direct discharge system with multiple electrodes in parallel.<sup>152–154</sup> The voltage drop across the electrodes was equivalent, although the current density was significantly lower, which reduced wear on individual electrodes. Additionally, this approach provided a larger treatment and mass transfer area. Johnson et al. improved this with the tubular high-density plasma reactor (THDPR), which addressed the problems of scale-up and continuous flow for industrial operations,

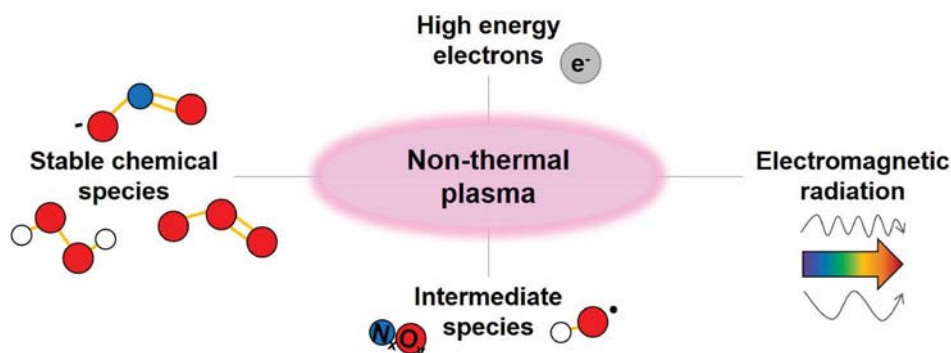
in addition to exposing reactive species created by plasma to a much larger volume of liquid.<sup>155</sup> The THDPR has demonstrated excellent disinfection capabilities, with the ability to inactivate bacteria with contact times on the order of seconds for power plant cooling tower water applications on the order of 500 V or less.<sup>156</sup> Another benefit to spreading the current density across multiple electrodes is the potential of reduced heat transfer, which is an important advantage for some possible plasma applications.

While surface-water systems have generally shown higher yields of reactive species produced by plasma on a  $\text{g kW}^{-1} \text{ hr}^{-1}$  basis, the nature of their design makes scale-up to industrial levels difficult or costly. Direct discharge methods, particularly point-to-plane with the direct injection of gas and modifications such as multiple electrodes in a cylindrical system, have the greatest potential for scaling up to a more useful level and also overcome diffusion limitations associated with above-solution discharges. Additionally, more recent innovations such as electrode coatings or multiple parallel electrode configurations promise to help mitigate longevity problems such as electrode wear.<sup>142</sup> Overall, many different electrical discharge methods are available for preparing plasma-activated solutions for subsequent therapeutic use. Such technologies enable plasma discharge above or directly in an aqueous solution, where oxidant plasma species can be transferred to the bulk solution. In subsequent sections, we describe in more detail which systems are being actively investigated; however, some of these systems have yet to be thoroughly investigated for APP applications.

### III. AQUEOUS PLASMA CHEMISTRY

The complex chemistry that occurs in aqueous plasma systems is reactive in nature and is dependent on factors such as feed gas composition, aqueous solution composition, power input, and time parameters (e.g., plasma exposure time and lifetime of solution post-preparation). The complexity of plasma-generated solutions with regards to oxidant species formation, composition, reactivity, and stability not only presents an analytical challenge but also sets APP apart as a unique and synergistic approach to tailoring oxidant chemistries such that different therapeutic processes can be targeted. As such, the tunable combination of oxidant species in solution enables plasma-activated solutions to target multiple cellular pathways that would not otherwise be achieved by a single oxidant species alone or non-plasma-generated mixes of multiple oxidant species. Many fundamental studies have been performed to determine the physical properties of individual plasma systems; the goal of this review, therefore, is not to review each characterization study in depth but to generalize such findings to be relevant to the field of APP. Several reviews are available that describe the chemical characterization of species prepared via electrical discharges in solution,<sup>143,144,157,158</sup> herein, we give a comprehensive overview of these findings.

In general, plasma discharges result in several different routes of action (Fig. 3), including (a) electromagnetic radiation (visible, IR, UV, EMF), (b) high-energy electrons, (c) intermediate chemical species (radicals, ions), and (d) stable chemical products (ions, low-molecular-weight products). The identity and quantity of these species is dependent upon



**FIG. 3:** Plasma discharges initiate multiple mechanisms, including electromagnetic radiation, high-energy electrons, intermediate chemical species, and stable chemical species.

variable system parameters, as discussed in more detail below. While all of these processes are important to determine which species form in solution during plasma treatment, the longer-lived oxidant species in solution will ultimately impact cellular function to impart therapeutic action. Thus, for APP applications, it is necessary to understand which plasma parameters will enhance the formation of species that are most therapeutically relevant.

The multitude of reactions possible in plasma plumes and subsequently in aqueous solution are initiated by electron processes. The high-energy electrons produced experience inelastic collisions with ambient molecules, atoms, and ions to yield multiple reaction effects, including excitation (Eq. 1), dissociation (Eq. 2), electron capture (Eq. 3), and ionization (Eq. 4).



For example, energetic electrons result in water dissociation to form  $\text{OH}^{\bullet}$  and  $\text{H}^{\bullet}$ , among other physical processes.<sup>159</sup> In nonthermal plasma, the plasma does not reach local thermal equilibrium, and high-energy electrons are present among non-excited atoms and molecules. The presence of energetic electrons and parent species ( $\text{O}_2$ ,  $\text{N}_2$ ,  $\text{H}_2\text{O}$ ) enables the formation of primary plasma species, such as radicals, that ultimately result in the formation of secondary species, namely reactive oxygen species (ROS) and reactive nitrogen species (RNS).

In general, species formed in the plasma include radicals ( $\text{O}^{\bullet}$ ,  $\text{OH}^{\bullet}$ ,  $\text{N}^{\bullet}$ ,  $\text{NO}_2^{\bullet}$ ,  $\text{NO}^{\bullet}$ ), excited state species ( $\text{O}_3^*$ ,  $\text{N}_2^*$ ,  $\text{N}^*$ ), cations ( $\text{O}_2^+$ ,  $\text{N}_2^+$ ,  $\text{N}^+$ ,  $\text{O}^+$ ,  $\text{NO}^+$ ), and anions ( $\text{OH}^-$ ,  $\text{O}_2^-$ ,  $\text{O}^-$ ).<sup>144</sup> Such species undergo interfacial transfer from the plasma phase to the liquid

phase, where subsequent chemistries occur within solution to yield species in the aqueous solution ( $\text{NO}_2^-$ ,  $\text{NO}_3^-$ ). Additionally, high-energy electrons formed in the plasma phase can penetrate into the aqueous solution phase to form solvated electrons.<sup>160</sup> The emission of UV light is due to the relaxation of excited state species, where the presence of UV light can result in subsequent reactions, such as the formation of  $\text{H}^\bullet$  and  $\text{OH}^\bullet$  from the photolysis of  $\text{H}_2\text{O}$ . Predictive models demonstrate that hydrogen ( $\text{H}_2$ ) formation can occur via hydrogen radical recombination.<sup>161</sup>

For electrical discharges directly in aqueous solution, a variety of reactions occur between water molecules and electrons, including numerous rotational excitation, vibrational excitation, dissociation, ionization, and attachment processes.<sup>158</sup> As such, direct aqueous discharges represent an attractive approach for the preparation of plasma-activated solutions. Electrical discharges produced directly in aerated water yield similar potent species ( $\text{OH}^\bullet$ ,  $\text{H}^\bullet$ ,  $\text{O}^\bullet$ ) as well as stable oxidant products, such as ozone ( $\text{O}_3$ ) and hydrogen peroxide ( $\text{H}_2\text{O}_2$ ). Combined, the oxidant species that form in plasma plumes and aqueous electrical discharges represent some of the most potent oxidant species due to their high oxidation potentials. Notably, the hydroxyl radical ( $\text{OH}^\bullet$ ) is discussed widely in the literature as being a critical player for *in situ* reactivity as it has a high oxidation potential compared to other plasma species; although short-lived,  $\text{OH}^\bullet$  is a critical species formed in the plasma-gas phase, which can diffuse to the aqueous phase or form via subsequent aqueous reactions.<sup>162</sup> Critical to solution reactivity is the presence of excess  $\text{H}_2\text{O}$ , which results in a series of reactions that are described in Table 3 along with pertinent rate constants as reviewed by Malik et al.<sup>144</sup> Overall, chemistries in plasma, gas, and aqueous phases, as generalized in Fig. 4, are versatile and dictated by interfacial diffusion processes. Considering the short lifetimes and high reactivities of the radicals and electrons formed, these processes occur on very short timescales.<sup>159</sup>

As a result of the complex chemistry ongoing in the aqueous phase during plasma discharge, many short-lived intermediates form, which ultimately result in the formation of stable end products, such as  $\text{H}_2\text{O}_2$  and  $\text{O}_3$ , which can go on to participate in oxidative effects for therapeutic or other applications. Ozone is formed predominantly due to the reaction of diatomic oxygen ( $\text{O}_2$ ) with oxygen radical ( $\text{O}^\bullet$ ) in the presence of a third collision partner, M (such as  $\text{O}_2$  or  $\text{N}_2$ ), according to Eq. 5.<sup>163</sup>



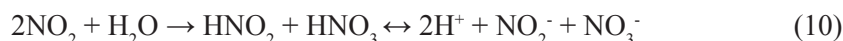
Hydrogen peroxide formation in nonaerated, nonoxygenated systems typically proceeds via hydroxyl radical combination (Eq. 6); under oxygenated conditions,  $\text{H}_2\text{O}_2$  formation is facilitated via hydroperoxyl radical recombination (Eq. 7).<sup>164</sup> Once formed,  $\text{O}_3$  and  $\text{H}_2\text{O}_2$  participate in a number of aqueous reactions that lead to additional species or can act directly on cells or biomolecules.



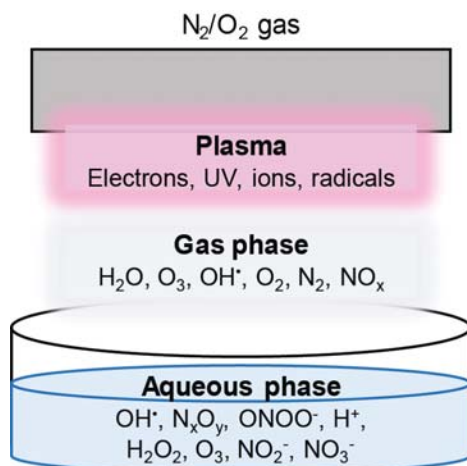
**TABLE 3:** Prevalent chemical reactions that occur during electrical discharge in aqueous solution and corresponding rate constants

Reaction	Rate constant ( <i>k</i> )
$\text{H}_2\text{O} \rightarrow \text{OH}^\bullet + \text{H}^\bullet$	$9.25 \times 10^{-10} \text{ M s}^{-1}$
$2\text{H}_2\text{O} \rightarrow \text{H}_3\text{O}^+ + \text{e}_{\text{eq}}^- + \text{OH}^\bullet$	$2.35 \times 10^{-9} \text{ M s}^{-1}$
$\text{H}_2\text{O} \rightarrow \frac{1}{2}\text{H}_2\text{O}_2 + \frac{1}{2}\text{H}_2$	$1.2 \times 10^{-6} \text{ M s}^{-1}$
$\text{H}^\bullet + \text{O}_2 \rightarrow \text{HO}_2^\bullet$	$1.0 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$
$\text{H}^\bullet + \text{H}_2\text{O}_2 \rightarrow \text{H}_2\text{O} + \text{OH}^\bullet$	$1.0 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$
$\text{H}^\bullet + \text{HO}_2^\bullet \rightarrow \text{H}_2\text{O}_2$	$1.0 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$
$2\text{H}^\bullet \rightarrow \text{H}_2$	$1.0 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$
$\text{H}^\bullet + \text{OH}^\bullet \rightarrow \text{H}_2\text{O}$	$2.4 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$
$\text{H}^\bullet + \text{e}_{\text{eq}}^- + \text{H}_2\text{O} \rightarrow \text{OH}^\bullet + \text{H}_2$	$2.5 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$
$\text{OH}^\bullet + \text{H}_2\text{O}_2 \rightarrow \text{H}_2\text{O} + \text{HO}_2^\bullet$	$5 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$
$2\text{OH}^\bullet \rightarrow \text{H}_2\text{O}_2$	$4.0 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$
$\text{OH}^\bullet + \text{HO}_2^\bullet \rightarrow \text{H}_2\text{O} + \text{O}_2$	$1.0 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$
$\text{OH}^\bullet + \text{e}_{\text{eq}}^- \rightarrow \text{OH}^-$	$3 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$
$2\text{HO}_2^\bullet \rightarrow \text{H}_2\text{O}_2 + \text{O}_2$	$2.0 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$
$\text{H}_2\text{O}_2 + \text{e}_{\text{eq}}^- \rightarrow \text{OH}^\bullet + \text{OH}^-$	$1.2 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$
$\text{H}_3\text{O}^+ + \text{OH}^- \rightarrow 2\text{H}_2\text{O}$	$3.0 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$

Additional reactions are possible due to the presence of  $\text{N}_2$  in systems that use air as the feed gas, ultimately resulting in production of  $\text{N}_x\text{O}_y$  species, both short-lived and stable. In plasma, nitrogen and oxygen gas combine to form nitric oxide (NO) according to Eq. 8. Further, NO reacts with  $\text{O}_2$  to form nitrogen dioxide ( $\text{NO}_2$ ) according to Eq. 9. In general,  $\text{NO}_x$  species formed in the air discharge will transfer to the aqueous phase, where reaction with water results in  $\text{NO}_2^-$  and  $\text{NO}_3^-$  accompanied by  $\text{H}^+$  formation, which results in pH drop, as shown in Eq. 10 and Eq. 11.



Under these acidic conditions,  $\text{HNO}_2$  undergoes further reaction to result in the formation of nitric oxide ( $\text{NO}^\bullet$ ), nitrogen dioxide ( $\text{NO}_2^\bullet$ ), nitrosonium ion ( $\text{NO}^+$ ), and



**FIG. 4:** Variable species are formed in the plasma, gas, and aqueous phases to ultimately result in a variety of aqueous plasma species that can be applied for therapeutic action.

other N<sub>x</sub>O<sub>y</sub> species (e.g., N<sub>2</sub>O, N<sub>2</sub>O<sub>3</sub>, and N<sub>2</sub>O<sub>4</sub>), including peroxynitrite (ONOO<sup>-</sup>) due to reaction of H<sub>2</sub>O<sub>2</sub> and HNO<sub>2</sub>.<sup>165</sup>

In general, many factors impact the aqueous plasma species that form in solution, including (1) the choice of feed gas (if used), (2) choice of plasma system and the system power input, (3) the solution properties (pH, conductivity, and composition), and (4) time parameters, as discussed in detail below. Such parameters can be manipulated to tailor the resultant solution chemistries for subsequent therapeutic application.

### A. Feed Gas

As described previously, OH<sup>•</sup>, H<sup>•</sup>, and O<sup>•</sup> production is enhanced for gas bubble configurations, where increased production correlated to increased rate of gas bubbling.<sup>144</sup> Additionally, H<sup>•</sup> is favored with argon as the feed gas, but minimized when oxygen gas is used, where O<sup>•</sup> is instead the major radical product. As such, to enhance the formation of RNS, N<sub>2</sub>-containing feed gas sources can be used, such as pure N<sub>2</sub> to maximize RNS formation, or house air, which contains both N<sub>2</sub> and O<sub>2</sub>. To enhance chemistries involving O<sup>•</sup> for ROS species formation, oxygen feed gas is suggested, whereas inert argon gas favors H<sup>•</sup> chemistries. The use of aqueous bubble discharge systems has yielded a wide range of H<sub>2</sub>O<sub>2</sub> generation rates, ranging from 0.002 to 2 g/h for Ar (pulsed corona and multi-electrode systems) to 0.01 g/h for air (DC discharge) and ~2 g/h for O<sub>2</sub> (pulsed systems).<sup>164</sup> For saline solutions containing sodium chloride (NaCl), a DC microplasma jet discharge resulted in predominantly sodium hydroxide (NaOH) formation in solution with oxygen or argon gas, whereas nitric acid (HNO<sub>3</sub>) formation dominated for air or nitrogen gas.<sup>148</sup> Thus, the use of O<sub>2</sub> or Ar gas can lead to alkaline solutions, whereas air or N<sub>2</sub> gas will yield acidic solutions. Overall, the feed gas can be tuned to achieve different



target therapeutic oxidant species that are favored for therapeutic action. For instance, to target a more highly oxidizing plasma-treated solution, oxygen or air feed gas can be used to favor ROS species, while nitrogen feed gas favors RNS.

## B. Plasma System and Power Input

In general, the concentration of primary reactive species formed in a plasma discharge, and subsequently the species formed in aqueous solution, is dependent upon the system energy input, but it must be balanced with the cost of power production and undesirable effects that may occur, such as thermal heating.<sup>143</sup> As highlighted in a prior review focused on water treatment, chemically active species in solution were increased by the following discharge parameters: (a) increasing applied voltage, but optimized in the 15–25 kV range; (b) decreasing the radius of curvature of the needle electrode, if a needle electrode configuration is employed; and (c) using positive polarity rather than negative polarity for DC voltage.<sup>144</sup> Ozone concentration increased with increasing applied voltage and gas residence time for a given  $N_2/O_2$  feed gas system; additionally, for a dielectric barrier (DB) system, ozone generation was increased when using a single DB versus double DBs.<sup>166</sup> Similar energy yields were required for  $H_2O_2$  production across multiple plasma platforms, including RF, pulsed, AC, and DC, thus suggesting a radical quenching rate-limiting step at the plasma-liquid interface; the energy yield was improved by employing discharges in bubbles, presumably due to overcoming diffusion limitations by increasing the surface area of the plasma-liquid interface.<sup>164</sup> Earlier studies employing a pulsed streamer corona discharge treatment of aqueous (DI water) solution indicated that the time-averaged power input greatly impacted the formation of plasma species; more specifically,  $OH^\bullet$  and  $e^-_{(aq)}$  concentrations increased in a nonlinear fashion with respect to average power input, while  $H_2O_2$  formation increased linearly.<sup>167</sup> Other studies indicate a linear increase in  $O_3$  concentration when using a point-to-plane discharge in solution up to ~20 kV, past which point a linear decrease in  $O_3$  concentration was observed.<sup>168</sup> Notably, certain plasma setups, such as plasma discharge above solution, experience diffusion limitations that limit the concentration of plasma species formed in solution. However, certain setups that maximize the surface area between the plasma region and the aqueous phase result in more facile transfer of species to solution, such as systems that involve plasma discharge into gas bubbles.<sup>151</sup> To maximize the oxidizing capacity of plasma-prepared solutions, the choice of plasma configuration and applied power is critical. For instance,  $O_3$  has been identified as a key species responsible for therapeutic activity, as described in later sections; thus,  $O_3$  levels can be increased by increasing applied voltage. As more is learned about the cellular mechanism of action and which species are most therapeutic, such parameters can be tuned accordingly.

## C. Solution Composition

In general, the solution conductivity and the presence of any solutes will greatly dictate the resultant chemistry in solution. The conductivity of the aqueous solution has been



shown to play an important role in the production of aqueous species.<sup>144</sup> More specifically, weak radical ( $\text{OH}^\bullet$ ) emission intensity was observed for low conductivity solutions ( $1\ \mu\text{S}/\text{cm}$ ), but the radical emission intensity and discharge properties strengthened and streamer channel length increased with an increase in the solution conductivity in the range  $10\text{--}80\ \mu\text{S}/\text{cm}$ , past which point the radical emission intensity weakened.<sup>151</sup> Additionally, in the same study, the choice of KOH over KCl as the solution ions resulted in stronger radical emission due to possible pH effects where  $\text{OH}^\bullet$  levels increase at neutral or alkaline pH. Other work has shown that, despite the use of different electrode geometries to facilitate corona discharge in solution, similar spectral features were generated for a given solution conductivity, thus implying that that solution chemistry may dictate aqueous plasma species more so than the choice of electrode configuration.<sup>169</sup> A recent review outlined the variable levels of  $\text{H}_2\text{O}_2$ ,  $\text{NO}_2^-$ , and  $\text{NO}_3^-$  formed in solution when exposed to a dielectric barrier discharge system at variable pH and salt content.  $\text{H}_2\text{O}_2$  levels were  $\sim 0.1\ \text{mM}$  for deionized (DI) water, phosphate-buffered saline (PBS) solution, and saline; however, higher concentrations of  $\text{NO}_2^-$  and  $\text{NO}_3^-$  formed in PBS at pH 6.5 versus DI water at pH 2.7.<sup>165</sup> Another review highlights several studies employing direct electrical discharges in solution, whereby the solution conductivity and pH along with power consumption led to variable levels of plasma species such as  $\text{H}_2\text{O}_2$  and  $\text{OH}^\bullet$ .<sup>158</sup> In the presence of saline solution containing NaCl, mechanisms have been deduced involving atomic oxygen reactions with chloride to yield  $\text{Cl}_2^\bullet$  or  $\text{ClO}^\bullet$  species, where the chlorine chemistries dominated  $\text{H}_2\text{O}_2$ -mediated reactions.<sup>170</sup> In the presence of solutes in the aqueous phase, variable oxidation reactions occur mainly due to  $\text{OH}^\bullet$  reactions. In general, it is critical to recognize how the parameters of the initial aqueous solution, such as composition and pH, will impact which species form. For instance, as described below, much APP work related to cancer treatment has involved activation of cell media, where subsequent stability issues have been recognized. It will be critical to determine whether the plasma activation is most effective before or after the addition of reagents, and whether components, such as cell nutrients, are actually required.

#### D. Time

A recent review describes the time scales associated with variable plasma processes, such as diffusion, reaction, thermal conduction, speed of light, and radiation, all of which are critical to the time-dependent formation of variable primary and secondary plasma species.<sup>157</sup> Aqueous concentrations of plasma-induced  $\text{H}_2\text{O}_2$  and  $\text{O}_3$  increased with discharge time until eventually a saturation level was reached.<sup>169</sup> For example, a study considering  $\text{O}_3$  production specifically indicated that  $\text{O}_3$  concentration in solution increased with increasing treatment time up to  $\sim 20\ \text{min}$ , past which point  $\text{O}_3$  generation was no longer favored and the  $\text{O}_3$  levels started to decrease due to purging of  $\text{O}_3$  into the gas phase via  $\text{O}_2$  bubbling. This observation was explained due to an increase in solution conductivity with increasing plasma treatment time due to dissolved ionic species that formed in solution; the increased conductivity of the solution thus reduced intensity of the spot discharge and increased streamer production, whereby  $\text{O}_3$  production was re-

duced.<sup>168</sup> Other studies show a linear relationship between oxidant concentration ( $\text{H}_2\text{O}_2$ ,  $\text{NO}_3^-$ ,  $\text{NO}_2^-$ ) and discharge treatment time for up to 30 min for an aqueous solution at pH 6.9 exposed to an air discharge plasma.<sup>165</sup> Further, concentrations of  $\text{H}_2\text{O}_2$ ,  $\text{NO}_3^-$ ,  $\text{NO}_2^-$  were monitored up to 300 s post-discharge time where, at lower pH (3.3),  $\text{H}_2\text{O}_2$  and  $\text{NO}_2^-$  levels dropped while  $\text{NO}_3^-$  levels increased; however, at higher solution pH, the oxidant levels remained constant post-plasma treatment. Other effects that are pronounced with increased plasma treatment include the acid effect, wherein a drop in solution pH occurs and can proceed on a rapid timescale (order of minutes).<sup>143</sup> The plasma exposure time is critical to determine which species form and in what concentrations; additionally, the length of time between solution preparation and application to a cell or tissue will dictate which species remain in solution for subsequent therapeutic activity.

A multitude of complex chemical reactions occur in plasma plumes and subsequently the plasma-treated aqueous solution. Many factors impact the identity and concentration of species that form in solution, as well as their lifetime, including choice of plasma system, feed gas, power input, composition of the aqueous solution, and time parameters. The use of aqueous plasma systems should enable a specific set of reactions that can be harnessed for the most optimized and effective water treatment or use for subsequent therapeutic applications in the case of APP. However, because so many factors impact the subsequent solution chemistry, it is common to achieve competitive rather than specific reactions.<sup>143</sup> Additionally, because the plasma chemistry is so diverse and complex, the ability to achieve specific chemical products can be difficult. However, using the aforementioned parameters as applied to water treatment, much can be learned about tuning solution chemistries for medical applications specifically. Notably, some of the plasma species discussed above have been characterized in different plasma plume and plasma-treated aqueous systems using various methods; however, much basic research is still needed to understand the complex reactivity and abundance of species present in activated solutions prepared by multiple different discharge methods.

#### IV. APP APPLICATIONS

Along with the development of methods for facilitating plasma discharges into solutions and increased understanding of the relevant chemical reactions involved, fundamental advances also have been made in the field of APP in recent years. More specifically, work in the past 10 years has focused on the preparation of plasma-treated bioactive solutions for applications in disinfection/antiseptics, cell proliferation/growth inhibition relating to angiogenesis and wound healing, and selective cancer therapies. In this section, we concentrate on three main APP research areas: (1) disinfectant solutions, (2) cell proliferation related to wound healing, and (3) cancer treatment.

##### A. Disinfectant Solutions

In general, the use of plasma discharges in water for direct *in situ* disinfection operates on the multimechanistic approach in which active oxidant species, UV, ozone, and hy-

drogen peroxide all act in concert to inactivate microbes.<sup>146</sup> In some studies,  $\text{H}_2\text{O}_2$  has been identified as a critical oxidant species due to its ability to interact with UV radiation and shock waves to form  $\text{OH}^\bullet$  radicals in bulk solution,<sup>171</sup> where  $\text{OH}^\bullet$  has been implicated as a major antimicrobial player.<sup>172</sup> Other work has suggested  $\text{O}_3$  as the dominant species responsible for disinfection,<sup>173</sup> while some studies suggest  $\text{H}_2\text{O}_2$  and acidified nitrite ( $\text{HNO}_2$ ) as key players.<sup>165,174–176</sup> One study found nitric oxides to be predominant long-lived species produced by plasmas for disinfection.<sup>114</sup> However, work involving the application of plasma-treated water to wounds or surfaces for disinfection will likely harness the action of multiple stable oxidant species in solution, and will be dependent upon the types and concentrations of oxidants formed depending upon the initial solution and plasma parameters employed.

The use of “plasma activated water” (PAW) for secondary disinfection was investigated by Naitali et al. through a series of studies employing gliding electrical discharges, shown in Fig. 1(a). In 2007, they studied both direct (*in situ* treatment of contaminated aqueous solutions) and indirect (preparation of PAW for *ex situ* secondary aqueous disinfection) methods of bacterial inactivation and found that plasma treatment time was critical for both configurations.<sup>177</sup> More specifically, for a given 20-min contact time for *Hafnia alvei* exposed to PAW, a longer plasma exposure period during initial PAW preparation yielded more effective secondary disinfection, such as a 2- or 10-min exposure time, resulting in 3.7- or 7-log disinfection, respectively. Follow-up work with *H. alvei* considered the impact of various exposure times using 5 min PAW as well as varying initial concentrations (CFU) of planktonic *H. alvei*.<sup>178</sup> First-order inactivation kinetics were observed, most likely rate-limited by mass transfer of the active disinfection species into solution, where longer treatment times yielded a greater log-reduction in CFU; further, the starting concentration of *H. alvei* was critical, where a decrease in initial CFU concentration yielded faster inactivation kinetics. Further, bacteria adhered to substrates were less susceptible to treatment compared to planktonic bacteria in solution, which follows expectations because biofilm bacteria are usually more difficult to treat. Additional work considered the use of PAW to treat various bacteria strains, including *S. epidermidis*, *L. mesenteroides*, *H. alvei*, and a yeast model, *S. cerevisiae*.<sup>179</sup> Again, treatment was generally more effective for the planktonic form of each strain compared to the adherent form; also, bacterial inactivation was more effective than yeast treatment. Another study implicated nitrite as a major species in PAW, while also considering a synergistic effect of nitrate and  $\text{H}_2\text{O}_2$ .<sup>180</sup> In other discussions, Naitali has implicated  $\text{ONOOH}/\text{ONOO}^\bullet$  as a critical part of the disinfection mechanism.<sup>181</sup>

Plasma-activated saline solution has also been investigated for secondary disinfection applications. More specifically, work out of Leibniz Institute indicated that a surface dielectric barrier discharge treatment of a NaCl solution yielded at least 7-log disinfection of *E. coli*, which was similar to the disinfection kinetics observed for directly treated *E. coli* in saline solution.<sup>182</sup> Further, a 30-min-old solution yielded less facile disinfection kinetics but still yielded >4-log reduction, thus indicating that the lifetime of the solution is an important parameter. This work further suggested  $\text{NO}_2^-$  and  $\text{H}_2\text{O}_2$

as the major stable species, but other species such as  $O_3$ ,  $ONOO^-$ ,  $OH^\bullet$ ,  $NO^\bullet$ , and  $NO_2^\bullet$  were suggested. While the lifetime of radicals is very short, some of these species may be available for cellular action via decomposition of other oxidant species in solution.

The shelf life of the PAW solution is critical to understand the stability of plasma species in solution and subsequent antibacterial effects. Plasma-activated solutions have been demonstrated to maintain long-term antibacterial efficacy on the order of days.<sup>183</sup> For a 3-h contact time, disinfection efficacy was maintained for 2-day-old PAW; however, efficacy dropped by  $\sim 1$  log by 4 days, and  $\sim 2$  log by 7 days. For a 15-min exposure time, however, efficacy dropped by  $\sim 5$  log for both 1- and 2-day-old solutions. Thus, disinfection efficacy can be maintained, but it is dependent upon contact time between PAW and *E. coli*. Over the course of the 7-day study, solution composition did vary in terms of  $NO_2^-$  and  $H_2O_2$  levels dropping, which correlated to loss of disinfection efficacy; however, likely different species exist on different time scales that still enable disinfection for 2+ day-old PAW solutions. In other work, plasma-activated solutions of water and saline exhibited antimicrobial effects after 4 weeks of storage, despite significant loss of initial  $H_2O_2$  and  $O_3$  by the end of the storage period.<sup>184</sup> Additionally, preparing solutions of similar oxidant concentration did not yield the same disinfection effects as the plasma-activated solutions, indicating that the versatile chemistry induced by plasma is required for optimum effects. Other studies have indicated that the ratio of aqueous  $NO_2^-$  to  $H_2O_2$  in addition to plasma discharge parameters are critical to establish long-term antibacterial effects of saline solutions.<sup>84</sup> To further extend the shelf life of PAW solutions, it has been demonstrated that the addition of *N*-acetyl-cysteine (NAC) prior to plasma treatment resulted in solutions with a shelf life of 2 years; however, the mechanism is unclear.<sup>185</sup> These results indicate the potential for these plasma-activated solutions to be prepared in advance for subsequent shipment, storage, and clinical use; however, if shelf-life limitations become apparent in future work, the commercial focus may shift to on-site generation for direct use upon preparation.

An additional benefit for using PAW is that these versatile oxidant-containing solutions are less prone to developing antibiotic resistance compared to traditional small-molecule antiseptics and antibiotics.<sup>84</sup> This approach has been proposed in light of the complex plasma species involved that suggest multiple modes of disinfectant action, where plasma treatment in general has been implicated for broad spectrum bacterial kill.<sup>186</sup> Antibacterial resistance is currently an overwhelming issue in the healthcare arena; thus, the development of plasma-activated aqueous solutions with the capability to address this problem and effectively reduce bacterial loads will be hugely beneficial.

Overall, the exact mechanism of PAW for disinfection is unknown. Variable plasma systems have been employed to prepare such solutions, and different species, such as  $H_2O_2$ ,  $NO_3^-$ , and  $NO_2^-$  have been implicated, along with importance of the acid effect. Disinfection has been demonstrated to be more effective using PAW versus mock solutions prepared with individual or mixed species, thus demonstrating the versatile nature of the plasma activation that leads to disinfection. These solutions also have demon-

strated stability and bactericidal properties even after up to 4 weeks of storage, despite significant drops in measured oxidant concentrations. Accelerated aging studies further indicate that the presence of certain additives can extend the shelf life on the order of years. In general, much is still unknown about the exact mechanism(s) involved and more work is required to understand how systematically to prepare optimized PAW solutions for the best disinfection results.

## B. Cell Proliferation Related to Wound Healing

The application of direct plasma plumes to dermal tissues has been of interest to promote wound healing. More specifically, plasma represents a synergistic approach to simultaneously reduce bacterial loads while also stimulating skin cells to initiate proliferation and angiogenesis to ultimately promote wound closure.<sup>88</sup> In addition to the dermal application of plasma plumes, plasma-activated aqueous solutions also have indicated the ability to promote similar effects, as discussed below, where an aqueous medium containing plasma species could be more effective at accessing internal sites within a wound to lead to more effective wound healing.

In 2010, work by Kalghatgi et al. described the effect of direct plasma treatment using a dielectric barrier discharge system on porcine aortic endothelial cells in the presence (50  $\mu\text{L}$ ) of medium to “prevent drying” of cells during treatment.<sup>40</sup> Low doses of plasma (4  $\text{J cm}^{-2}$  at up to 30 s treatment time) yielded cell proliferation after treatment, whereas higher plasma doses (8  $\text{J cm}^{-2}$  at  $\geq 60$  s treatment time) yielded cell death. Furthermore, fibroblast growth factor-2 (FGF2) was enhanced after plasma treatment, likely due to ROS-mediated FGF2 release, thus yielding proliferative effects. This study demonstrates the direct correlation between treatment time and ROS concentration, thus yielding proliferative or inhibitive cellular effects related to angiogenesis applications.

Work by Hamaguchi used a low-frequency plasma jet with He feed gas, where the tip of the plasma jet was just in contact with the surface of 100  $\mu\text{L}$  aliquots of Dulbecco's Modified Eagle Medium (DMEM) in a 96-well plate format.<sup>187</sup> The effects of plasma-treated DMEM on human synovocyte (HS) cells was explored in three different scenarios: (1) direct plasma exposure of DMEM containing cultured cells; (2) direct plasma exposure of DMEM containing adhered cells, whereby the media was immediately exchanged for fresh, non-plasma-treated DMEM; and (3) direct plasma treatment of DMEM only, followed by culture of cells in the plasma-treated medium. In each case, an optimized treatment time was found that would enhance cell proliferation (on the order of 60 s); however, plasma overexposure resulted in cell death. In general, a correlation between cell proliferation and solution oxidative stress was found where a subsequent simulation model indicated that an increase in ROS/RNS species (such as  $\text{OH}^{\cdot}$  and  $\text{NO}^{\cdot}$ ) directly affected HS proliferation. As such, when considering the variety of solution chemistries that can be tuned by various plasma parameters (as discussed above), it is beneficial to target oxidant levels that will be beneficial to kill bacteria, while not harming healthy cells, similar to the below discussion regarding selective cancer cell treatment. Thus, for a given application, such as wound healing, target oxidant levels must



be determined that will induce therapeutic effects while avoiding cell-damaging effects.

Earlier work at the Leibniz Institute by Hoentsch et al. considered the effects of both direct and indirect argon plasma jet aqueous exposure of murine epithelial cells (mHepR1).<sup>188</sup> For direct treatment, mHepR1 cells were suspended in cell culture medium (DMEM) for various plasma exposure times (30, 60, and 120 s), whereas the indirect treatment involved plasma treatment of DMEM alone (30, 60, and 120 s), followed by mHepR1 culture in the plasma-treated medium. The effects of plasma treatment times were assessed via cell viability and morphology. After only 30 s of mHepR1 direct plasma treatment in DMEM, significant morphological changes were observed, whereby the cells were no longer adherent thus inhibiting monolayer formation; similar effects were found for indirect plasma-activated DMEM treatment alone for 30, 60, and 120 s before cell exposure. For both direct and indirect methods, an increase in plasma exposure time yielded lower cell viability, where effects were more pronounced for the direct method. In general, cellular impacts of plasma-treated media were established concerning impact on morphology, viability, and tight junction formation, but no analysis of the aqueous species was performed to inform which radicals or compounds were responsible for these effects. These results point to the need to better understand the relationship between aqueous plasma species in solution and cellular effects; if, for instance, direct plasma treatment of epithelial cells in media diminished cell viability, this level of plasma exposure could be detrimental to the epithelium and to wound healing suggesting that milder conditions would be more suitable. As a follow-up to this work, studies also were published considering the stability of plasma-treated DMEM for subsequent application to mHepR1 cells.<sup>81</sup> Plasma-treated DMEM effects of changing cell morphology, viability, and tight junction formation were found for solutions even when stored for up to 7 days at 37°C prior to cell exposure *in vitro*. To inform the impact of medium composition on cells, pH, H<sub>2</sub>O<sub>2</sub>, and O<sub>2</sub> were assessed immediately after plasma treatment and after 1 day of storage. Overall, the pH dropped slightly but remained rather consistent after storage, whereas H<sub>2</sub>O<sub>2</sub> levels decreased dramatically (by ~90%) after 24 h, and O<sub>2</sub> levels in solution increased after storage. Based on these findings, it was assumed the RNS/ROS are critical to the bioactivity of the plasma-treated DMEM, but due to a sharp drop in H<sub>2</sub>O<sub>2</sub> levels after storage but maintenance of the cellular effects, it was assumed the species other than H<sub>2</sub>O<sub>2</sub> are critical to impact the cellular effects observed. Additionally, the observed effects on cellular morphology and viability are not desirable for wound healing applications. Thus, this suggests the need to further identify the exact composition of the solution and understand which configurations, such as <30 s of plasma treatment, will yield a solution that does not yield undesirable effects. However, this work was critical to establish the prolonged therapeutic effects that are possible for plasma-treated media even after storage for up to 1 week.

Further work by Winter et al. of the Leibniz Institute indicated that the concentration of H<sub>2</sub>O<sub>2</sub> in the liquid phase after plasma treatment via an atmospheric pressure argon plasma jet correlated directly with the viability of adhered human skin cells.<sup>80</sup> Complete cell-growth medium (RPMI) was plasma treated in a 60-mm-diameter Petri dish at vari-

able treatment times ranging from 0 to 100 s, where the plasma jet was moved constantly across the medium surface at a nozzle-liquid distance of 9 mm. Additionally, the rate of  $\text{H}_2\text{O}_2$  production in the gas phase matched that of net  $\text{H}_2\text{O}_2$  production in the liquid phase, where the  $\text{H}_2\text{O}_2(\text{g})$  solubility in the liquid phase was a key step. Other species studied by laser induced fluorescence (LIF) and electron paramagnetic resonance (EPR) spectroscopies indicated that  $\text{OH}^\bullet$  and  $\text{O}_2^{\bullet-}$  did not vary significantly with treatment, thus indicating  $\text{H}_2\text{O}_2$  as a critical species in initiating proliferation of skin cells. Due to its longer half-life in solution compared to other shorter-lived species such as  $\text{OH}^\bullet$  or  $\text{O}_2^{\bullet-}$ ,  $\text{H}_2\text{O}_2$  is able to remain in solution on the timescale required for direct cellular exposure. In this study,  $\text{H}_2\text{O}_2$  concentrations in plasma-treated RPMI solution were achieved at 0.8, 2.9, and 9.0 mg/L due to variations in argon gas humidity and treatment time.  $\text{H}_2\text{O}_2$  levels were maintained for up to 1.5 hours post-treatment regardless of initial  $\text{H}_2\text{O}_2$  concentration; however, past 1.5 hours  $\text{H}_2\text{O}_2$  concentration decreased based upon the starting concentration. The addition of  $\text{H}_2\text{O}_2$  stabilizers significantly increased the stability of the  $\text{H}_2\text{O}_2$  in solution. Despite experimental evidence indicating  $\text{H}_2\text{O}_2$  as having a major role in initiating cell proliferation, additional species are also involved, but they were not quantified in this work. This research with human skin cells, wherein proliferative effects were observed, contrasts sharply with the Leibniz research in which plasma-treated media imparted undesirable morphological and viability effects, including loss of cell adhesion ability, for murine epithelial cells. Thus, these findings demonstrate the dependence of therapeutic effects on the cell line investigated, treatment time employed, and specific parameters of the system that will result in different plasma species in solution. Thus, a more thorough understanding of the aqueous plasma species and resultant solution chemistry is required to enable tailoring of the solution properties for a given application, as with traditional medicine approaches. While further exploratory work in the field is required, current findings also suggest a wide range of possibilities for future uses of plasma techniques for APP applications.

Reuter et al. further considered the impact of feed gas and ambient gas humidity on the aqueous plasma species formed and subsequent impact on human skin cells (HaCaT keratinocytes).<sup>189</sup> In general, ozone levels decreased with increasing humidity, whereas  $\text{H}_2\text{O}_2$  levels linearly increased with increasing humidity. The impact on HaCaT viability was unaffected based on varying humidity in the shielding gas, but a significant decrease in viability was found for increasing humidity in the feed gas. Specifically, for the feed gas humidity, the loss of HaCaT viability was attributed to HaCaT viability to decreased ozone and increased  $\text{H}_2\text{O}_2$  production, thus implicating  $\text{O}_3$  and  $\text{H}_2\text{O}_2$  as critical aqueous plasma species that impact skin cell viability. Earlier Leibniz work, also with HaCaT cells, indicated that direct cell treatment in the presence of medium to reduce cell viability was accompanied by a significant downregulation of E-cadherin and epidermal growth factor receptor (EGFR), with a lesser impact on  $\alpha_2$ - and  $\beta_1$ -integrins, and no impact on intercellular adhesion molecule 1 (ICAM-1).<sup>190</sup> Overall, this work informs the impact of plasma treatment on cell-surface adhesion molecules that are critical to wound healing.



In general, work on cell proliferation due to plasma-activated solutions has indicated that different routes of treatment can be employed (direct v. indirect), and that stable species, such as  $\text{H}_2\text{O}_2$  and  $\text{O}_3$ , are dominant in the resulting aqueous chemistry. Choice of feed gas, feed gas humidity levels, and treatment time are critical parameters to tune to achieve optimum proliferation related to wound healing and dermal applications. However, much remains unknown regarding the exact cellular mechanisms and all of the critical aqueous plasma species involved. Future systematic studies are required to determine how given plasma systems can be tuned to achieve chemistries that target specific desired cellular pathways.

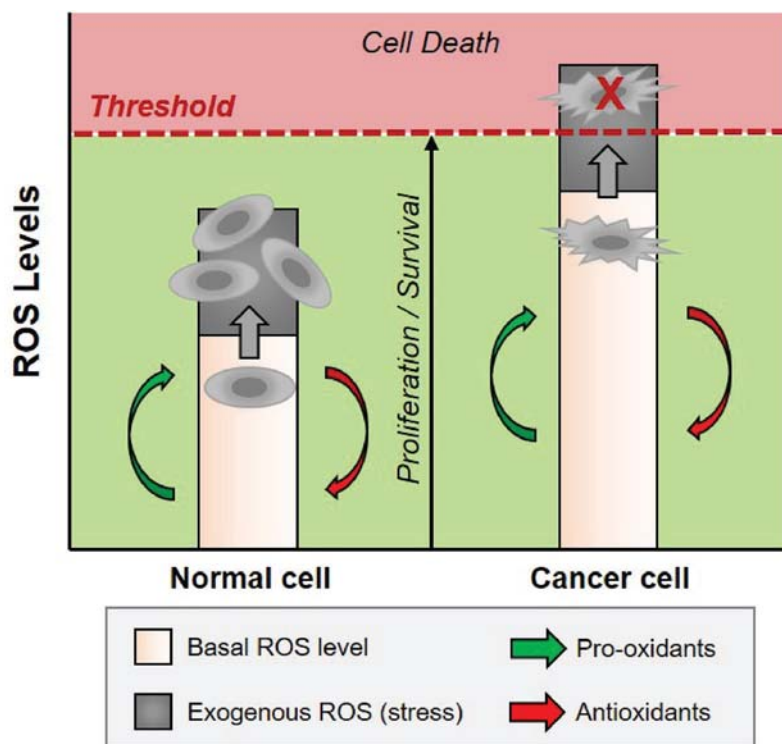
### C. Cancer Treatment

The clinical application of plasma species is of particular interest for selective cancer treatment. Despite significant advances in chemotherapeutic approaches over the past several decades with the advent of new drugs and advanced combinatorial therapies,<sup>191</sup> cancer is still the second leading cause of death in the United States.<sup>192</sup> A recent review highlighting emerging cancer therapies points out several limitations to current chemotherapeutic approaches in addition to limited efficacy, such as pathogenesis complications, drug resistance, cytotoxicity to normal healthy cells and tissues, side effects, inadequate delivery methods to the tumor site, and high recurrence rates of certain cancer types.<sup>193</sup> As such, all of these combined factors imply the critical need for new multi-functional cancer treatments to overcome issues associated with traditional treatment options. The success of any new cancer treatment depends on its ability to selectively target cancer cells while minimizing cytotoxicity to normal cells; plasma-related treatment may be an effective and unique solution.

For plasma plume direct treatment of cancer and normal cells *in vitro*, many different cancer types have been considered, including cervical,<sup>24</sup> colon,<sup>27,132</sup> glioblastoma,<sup>132</sup> head and neck,<sup>30</sup> oral,<sup>28</sup> liver,<sup>33</sup> breast,<sup>42,49</sup> bladder,<sup>50</sup> pancreatic,<sup>75</sup> prostate,<sup>127</sup> and ovarian cancers,<sup>97</sup> as well as melanoma<sup>32,34,129,135</sup> and leukemia.<sup>111,116</sup> General direct plasma plume work has indicated both cancer cell apoptosis and necrosis, where necrotic pathways were implicated at higher exposure times.<sup>111,116</sup> However, most studies have indicated apoptotic mechanisms that involve oxidative stress, DNA damage,<sup>27,41,89,118,132</sup> and mitochondrial dysfunction.<sup>24,30,118</sup> These references, and others cited in Table 1, have generally indicated that the involvement of ROS and RNS result in the following effects on cancer cells: cell detachment, inhibition of cell growth, impact on cell migration and invasiveness, clonogenicity, dose-dependent induction of apoptosis and/or necrosis, cell cycle arrest, senescence, induction of intra- or extra-cellular ROS production, and down-regulation of integrins. In general, the extent of therapeutic anti-cancer effects has been directly correlated to the plasma treatment time as the most critical parameter, although the exact mechanism is unknown. It has been suggested that the plasma species, such as  $\text{H}_2\text{O}_2$  and  $\text{NO}^\bullet$ ,<sup>35,194</sup> are able to interfere with the cancer cell-cycle regulation, as well as result in DNA damage, apoptosis, and cell membrane damage.<sup>18</sup> Due to the variable ROS/RNS and subsequent routes of action, it has been suggested that the use of

plasmas for cancer treatment is less likely to result in the development of drug resistance compared with other approaches.<sup>195</sup>

As discussed in a thorough review by Trachootham et al., the nature of oxidative stress on cancer cells, as induced by various ROS, is critical for the selective treatment of cancer cells.<sup>196</sup> As shown in Fig. 5, normal cells maintain redox homeostasis through a balance of ROS generation via pro-oxidants and elimination via antioxidants to maintain basal ROS levels for proper physiological function. In general, moderate increases in ROS levels can promote helpful processes, such as cell proliferation and differentiation. However, too much ROS, as represented by the threshold value, will impart oxidative damage to lipids, proteins, and DNA, leading to toxic cellular effects. The quantitative values associated with the ROS levels that are damaging to cancer cells but innocuous to normal cells vary depending upon the specific cell types and the identity of the ROS involved. Thus, the y-axis in Fig. 5 conceptually depicts ROS levels from low to high, with a threshold above which anti-proliferative effects are observed for cancer cells but not for normal cells. Normal cells can be exposed to a given amount of exogenous ROS



**FIG. 5:** The basal ROS levels are higher for cancer cells compared to normal cells, thus application of exogenous ROS species in plasma-activated solutions will push cancer cells over the ROS threshold, while normal cells are able to maintain homeostasis via antioxidant pathways. The actual ROS threshold level will vary depending upon the cell type and specific ROS involved.

stress due to reserves of antioxidants to prevent cellular ROS levels from reaching the fatal threshold. Cancer cells already exist at an increased ROS generation due to metabolic abnormalities and oncogenic signaling which is believed to lead to many cancer characteristics, including cancer cell proliferation, disruption of cell death signaling, and chemoresistance. When both cancer and normal cells are exposed to exogenous ROS levels, normal cells are able to maintain homeostasis, whereas cancer cells are pushed past the ROS threshold. Overall, cancer cells are more vulnerable to external oxidative stress than normal cells, thus enabling selective cancer cell destruction in the presence of oxidative species, such as those provided by a plasma-treated aqueous solution. Key to the investigation of plasma-induced anti-cancer applications will be the identification of the threshold ROS values required to selectively target cancer cells while leaving normal cells unharmed, which will likely vary depending upon the specific type of cancer. Such ROS-mediated approaches to targeting cancer treatment may overcome issues with traditional chemotherapies, such as cytotoxicity and chemoresistance.

In general, direct plasma plume treatment has significant clinical limitations as its geometry makes it most suitable for treating surface sites, such as skin cancer. One review describing direct plasma applications for prostate cancer specifically proposes delivery of plasma plume species to a tumor site transperineally with an accompanying transrectal ultrasonography (TRUS) probe.<sup>19</sup> However, this is a relatively complex mode of access to the tumor site, and one can imagine that general application of such a plasma plume approach to other types of internal cancer sites may be even more difficult compared to a prostate tumor. On the other hand, transfer of plasma species to the aqueous phase can create bioactive solutions that are more readily administered, even to difficult-to-reach places, such as internal tumors, or injected into the bloodstream for treating leukemia. Studies have indicated that plasma-activated aqueous solutions can perform just as effectively as direct application of plasma plume to cancer cells, and it has been further suggested that the transfer of plasma species to aqueous solution actually accesses certain chemistries and thus therapeutic effects that would not otherwise be possible with direct plasma plume treatment alone.<sup>23</sup> Additionally, plasma-treated liquids may be safer because they avoid direct tissue exposure to potentially undesirable plasma mechanisms, such as UV irradiation.

Initial APP research related to cancer treatment centered on exposing cell-containing solutions to plasma to understand the subsequent aqueous effects on cells. For example, a 2012 study by Graham et al. considered the impact of plasma-treating medium covering adhered breast cancer cells (Fig. 1, Scenario 2a), where DNA damage impacted cell viability in a dose-dependent manner.<sup>122</sup> Other work led by Old Dominion University considered treating free-floating media suspensions of leukemia cells (Fig. 1, Scenario 2b) to induce cell morphology and viability results in a dose-dependent manner.<sup>107</sup> However, as mentioned, the true goal of APP is to transfer plasma species to solution for subsequent application to cells or tissues. As such, below we present recent studies pertinent to the field for cancer treatment specifically. Table 4 highlights the major studies reviewed herein that focus on the plasma activation of cell media for secondary indirect

**TABLE 4:** Summary of the various studies considering plasma-activated media solutions for cancer treatment, both *in vitro* and *in vivo*

Institute	Year	Cancer Cell Line	Normal Cell Line	Result	Ref.
Nagoya University	2011	Glioblastoma, human (U251SP)	Normal brain astrocytes, human (ACBRI-371)	Selective apoptosis (caspase 3/7), AKT kinase down-regulation	95
	2012		Normal fibroblasts, human (WI-38)	Down-regulation of survival/proliferation signaling networks, selectivity	96
	2013	Ovarian cancer (paclitaxel/cisplatin resistant)	N/A	Anti-tumor effects demonstrated, <i>in vitro</i> and <i>in vivo</i>	98
	2014	Ovarian clear cell carcinoma (TOV21G, ES-2, SKOV3)	Peritoneal mesothelial cells, human	CCC selective apoptosis	99
	2015	Lung (A549), liver (HepG2), and breast (MCF-7) cancer lines	N/A	Media composition effects; apoptosis (mitochondrial dysfunction)	100
	2015	Gastric cancer (NUGC4, CS-2-NU, MKN28, MKN45)	Normal fibroblasts, human (WI-38)	Selective apoptosis (caspase 3/7); cell line effects	101
	2015	Pancreatic cancer (PANC-1, Capan-2, BxPC-3, MIA PaCa-2)	Normal pancreatic duct epithelial cells, human (HPDE6/C7)	Selective apoptosis (caspase 3/7, cell morphology); <i>in vitro</i> and <i>in vivo</i>	103
	2015	Melanoma (SK-Mel-147)	N/A	Cell migration and adhesion more affected than apoptosis	83
	2014	Glioblastoma, human (U87)	N/A	FBS concentration and temperature storage effects	61
George Washington University	2015	Glioblastoma (U87) and breast cancer (MDA-MB-231, MCF-7)	N/A	Potency based on cell line, optimized plasma treatment parameters, cysteine involvement	66
	2016	Glioblastoma (U87), pancreatic, and (PA-TU-8988T) breast (MDA-MB-231) cancers	N/A	Amino acid scavenging of ROS and implications on potency and storage	67

application of plasma species to cancer cells, which have been investigated only within the past five years.

The term “plasma-activated medium” or “PAM” was coined by researchers at Nagoya University’s Plasma Nanotechnology Research Center. Tanaka et al. have utilized a non-equilibrium atmospheric pressure plasma plume to expose cell media in a well plate format. In their seminal 2011 paper, PAM was demonstrated to selectively kill glioblastoma cells by inducing apoptosis via a caspase 3/7 pathway.<sup>95</sup> Additionally, the application of PAM to the cancer cells resulted in the downregulation of AKT kinase, thus impacting the survival signal transduction pathway. Cell viability also correlated with plasma treatment time, where lesser survival was found for a 5-min treatment time versus 1 min. No impact was observed on the normal brain astrocyte control line employed, suggesting selective cancer treatment. In terms of shelf life, the PAM lost its anti-tumor effects after 18 h, where the growth effects of the medium were restored, suggesting that it had no major impact on the composition of the growth medium. The limited 18-h window, past which point the solution lost its potency, suggests that on-site production of the PAM may be necessary for commercialization and clinical use. A follow-up publication in 2012 explored the mechanism of action against glioblastoma cells in more detail; major findings indicated complete downregulation of survival and proliferation signaling networks (e.g., AKT, ERK, and mTOR survival/proliferation pathways; CD44 membrane-bound receptor), while the control fibroblasts were comparably unaffected.<sup>96</sup> Other work has indicated similar findings for paclitaxel/cisplatin-resistant ovarian cancer cells, where the addition of *N*-acetyl-cysteine inhibited antitumor effects, presumably due to ROS scavenging, thus implicating ROS as the main players.<sup>98</sup> This study explored both *in vitro* effects as well as the treatment of xenografted tumors in an *in vivo* nude mouse model, where significant reduction in tumor growth was exhibited. Further work indicated the selective PAM treatment of ovarian clear cell carcinoma, which is particularly unaffected by conventional chemotherapies.<sup>99</sup>

Recently, three additional studies were published by the Nagoya team. In one study, the effects of PAM on lung, liver, and breast cancer lines were investigated based upon variations in the medium composition.<sup>100</sup> Pyruvate presence in the media decreased ROS availability, leading to less effective antitumor effects, where  $\text{H}_2\text{O}_2$  was identified as a critical player in apoptosis. In contrast to the 2011 glioblastoma work, this study indicated a caspase-independent apoptotic pathway, where mitochondrial stress and membrane effects were instead indicated due to  $\text{H}_2\text{O}_2$  and other reactive species. Furthermore, PAM maintained its potent therapeutic effects after 1 week of storage at  $-80^\circ\text{C}$ , thus indicating the possibility of storing bioactive solutions for later administration. Because  $\text{H}_2\text{O}_2$  was identified in this study as a critical oxidant species, storage of the solution at extremely low temperature ( $-80^\circ\text{C}$ ) in the dark would facilitate stabilization. Another 2015 study considered the impact of PAM on the cell morphology and proliferation rate of gastric cancer cells.<sup>101</sup> The impact on cancer cells was dependent upon plasma exposure time during PAM preparation, the cell line, and the initial cell concentration. Some cell lines were more resistant to PAM treatment, but this could be overcome by increasing the

plasma treatment time during preparation. In this work, the caspase 3/7 pathway was activated by PAM, leading to selective apoptosis; glutathione synthesis was also implicated. Caspases represent a family of protease enzymes that are essential to programmed cell death, including either initiator or executioner roles in apoptosis. The most recent Nagoya study investigated four different pancreatic cancer cell lines *in vitro*, where apoptosis was implicated via morphological changes and caspase 3/7 activation.<sup>103</sup> Additional *in vivo* studies using a xenograft mouse model demonstrated significant tumor reduction (similar to their 2013 study) upon subcutaneous injection of PAM at the tumor site. The Nagoya team has indicated that further research is required to understand the exact mechanism of action by which PAM is effective against cancer cells.<sup>104</sup>

Other work has investigated the plasma treatment of cell media to prepare bioactive solutions for administration to cancer cells. For example, Schmidt et al. recently reported that, more so than metabolic, apoptotic, and cell-cycle effects, plasma-treated medium impacted tumor cell motility and colony formation.<sup>83</sup> Gene analysis implied disorganization of the actin cytoskeleton via multiple signaling pathways, where interestingly genes related to cell adhesion, structure, and cell junction activity were more highly affected than those related to apoptotic signaling.

Work from the Keidar group at The George Washington University has examined the use of cold atmospheric plasma (CAP) to treat media, referred to as “plasma-stimulated medium” (PSM). They describe the PSM as a cocktail of various reactive species, which selectively targets cancer cells. One study indicated effects on cancer cell treatment due to the levels of fetal bovine serum (FBS) in the media.<sup>61</sup> In general, increased levels of FBS in solution increased the cell viability when exposed to PSM, while, for a given FBS concentration, increased plasma treatment time decreased cell viability. It was therefore concluded that the presence of FBS plays a protective role for the cancer cells. Considering the known effect of plasma species on amino acids (e.g., preferential reactions that decrease sulfur-containing and aromatic amino acids),<sup>197</sup> it is possible that the plasma treatment of media will result in ROS scavenging during treatment due to higher FBS levels, which will result in lower levels of ROS ultimately available to cells during exposure. Additionally, plasma impacts on the proteins and additives in solution could subsequently impact availability of cell nutrients.

Other work by Yan et al. demonstrated that PSM targeted both glioblastoma and breast cancer cells, where glioblastoma cells were more resistant to treatment due to faster consumption of effective reactive species.<sup>66</sup> It was suggested that  $H_2O_2$  is a critical ROS in PSM responsible for anti-cancer effects. Key to this work was a systematic review of several operating parameters during PSM preparation that optimized the potency of the resultant solution. More specifically, increasing the volume of media during treatment yielded lower ROS/RNS levels, whereas using a larger well for a given medium volume enhanced solution potency, presumably due to an increase in the interfacial surface area between the plasma plume and medium volume during treatment which enhanced plasma-to-liquid transfer of species. Similarly, holding the plasma plume closer to the medium during treatment enhanced PSM potency, again due to interfacial transfer



effects. Another key feature of this work considered which amino acid residues would consume relevant plasma species required for subsequent therapeutic action of PSM. Of the 20 amino acids studied, cysteine and tryptophan exhibited the strongest reactivity towards PSM plasma species, and cysteine prevented anti-cancer effects the best. Follow-up work identified methionine in addition to cysteine as medium-containing amino acids that scavenged plasma species most effectively.<sup>67</sup>

Additional effects of temperature during storage preadministration to glioblastoma cells have been considered.<sup>61</sup> PSM was held at room temperature (RT) or frozen (-20°C) for 1 and 2 days. For PSM supplemented with FBS prior to plasma treatment, no significant difference in cell viability between RT and -20°C stored PSM was found, but 1 day stored had more impact on cancer cells than 2 days. For media not supplemented with FBS and stored for 2 days, RT PSM yielded better anti-cancer effects than -20°C stored PSM. This is in contrast to the earlier described Nagoya work where freezing (-80°C) the PAM solution significantly increased the shelf life. However, it must be considered that the composition of media varies widely depending upon the cells that are being cultured; thus, it is expected that aqueous chemistries, and thus stabilities, will vary among media types and additives. More recently, methods for stabilizing PSM were considered in relation to amino acid scavenging effects.<sup>67</sup> The use of phosphate-buffered saline (PBS) or cysteine/methionine-free DMEM enabled anti-cancer effects after 3 days of storage at 8 and -25°C, whereas the addition of a tyrosine derivative to the unmodified DMEM enabled stable storage for 3 days at 8°C. These experiments have widespread applications to the general shelf life and storage considerations of plasma-activated solutions. Overall, the storage considerations for plasma-activated solutions are complex and application specific, and they depend upon solution generation parameters. Some points are indicated by prior studies, such as the elimination of cysteine and tryptophan from PAM formulations to minimize ROS reactivity; however, additional studies are required to understand how stabilization of these solutions can be achieved to enable shelf lives that are commercially relevant.

Overall, the use of plasma-activated solutions for subsequent application to cancer cells (*in vivo* or *in vitro*) has been indicated to yield selective antitumor effects across a range of cancer types and has been attributed to ROS effects. In general, the potency of the bioactive solution is related to plasma exposure time, but the effects also vary by cell line. Preliminary knowledge has been gathered about the mechanism of action due to apoptosis; however, much work is still required to identify the exact mechanism(s) involved based upon differences in the plasma treatment, aqueous solution composition, and cancer cell line of interest. Additionally, stability and subsequent anti-cancer effects are dependent upon the amino acid compositions of the media, and temperature also has an impact on the stability of the solution during storage.

## V. FUTURE DIRECTIONS IN APP

Together, much of the aforementioned research has highlighted the importance of multiple aqueous plasma species that can result in subsequent therapeutic action. The combination



of plasma species appears to be more potent than any individual chemical component or mixtures of certain compounds when added versus being plasma generated, which highlights the importance of such a synergistic, multifaceted therapeutic approach. Because much is still unknown about the exact mechanism of action for applications in disinfection, cell modification, and cancer treatment, it is critical to identify these species and subsequently understand which are most critical to the end application such that plasma chemistry can be tuned to favor the species and solution conditions that are most effective.

A recent book chapter by von Woedtke discussed one of the main hurdles in the field of plasma pharmacy, the ability to measure the aqueous plasma species with accuracy.<sup>174</sup> For plasma-prepared bioactive solutions to succeed in commercial applications, it will be critical to identify exactly the composition of these solutions for their subsequent therapeutic use. This research will require advanced analytical methods that are selective and sensitive for a given species of interest, thus enabling a detailed understanding of the plasma-solution mechanisms that give rise to therapeutic effects. The quantification of an oxidant species, such as  $\text{H}_2\text{O}_2$ , in the presence of other oxidant species, such as  $\text{O}_3$ , is compounded in difficulty for this application due to the multiple transient species, including radicals and short-lived intermediates, which may be present in the plasma-activated solution as a function of solution lifetime. Thus, the analytical methods of choice must be performed to capture an understanding of these system complexities while being selective and sensitive. A thorough understanding of solution composition, mechanism of action, and therapeutic indications will be critical to pass regulatory review, such as via the U.S. Food & Drug Administration (FDA). The aqueous chemistry will be heavily dependent upon the initial solution composition. Most of the work to date has been performed with saline solutions or well-established cell media formulations; it will be of great interest to explore additional aqueous formulations, such as plasma treatment of water alone where cell nutrient additives and additional reagents are added post-treatment. The identity of solution additives and whether they are added pre- or post-plasma treatment will greatly impact solution chemistry.

Part of understanding the exact chemistries and mechanisms of action will be a more thorough evaluation of different aqueous plasma discharge methods, as discussed in section II of this review. More specifically, most of the PAM/PSM studies related to cancer have been performed using liquid surface plasma plume exposure. Instead, however, it may lead to more promising chemistries and more effective therapeutics to use methods that directly discharge plasma into solution, with or without bubbles. Exploring various plasma methods and devices also will require thorough plasma diagnostics to understand the electrical, optical, thermal, and chemical properties of each system and subsequent impact on aqueous chemistry and the interfacial transfer kinetics that impact the concentration of plasma species that make it to the aqueous phase. The development of various commercial devices for generating plasma-activated solutions also will require attention to regulatory aspects.

An additional hurdle for the field will be to develop commercialization strategies that enable aqueous plasma systems and/or solutions to be available for clinical use.

Such strategies can include off-site generation of activated solutions, which can be shipped and stored prior to use. For this approach to work, much more work is needed to understand the stability of aqueous plasma species and the time effects on the solution composition and subsequent therapeutic efficacy for a number of applications. This includes a better understanding of preparation and storage conditions that will enable longer shelf life of the aqueous solutions such that they can be stored and shipped to clinicians. Another obstacle to commercialization is the small scale of current plasma-activated solution production methods. The creation of systems that can treat higher volumes of solution will be critical to producing plasma-activated species on a useful scale, which requires a better understanding of the plasma physics.

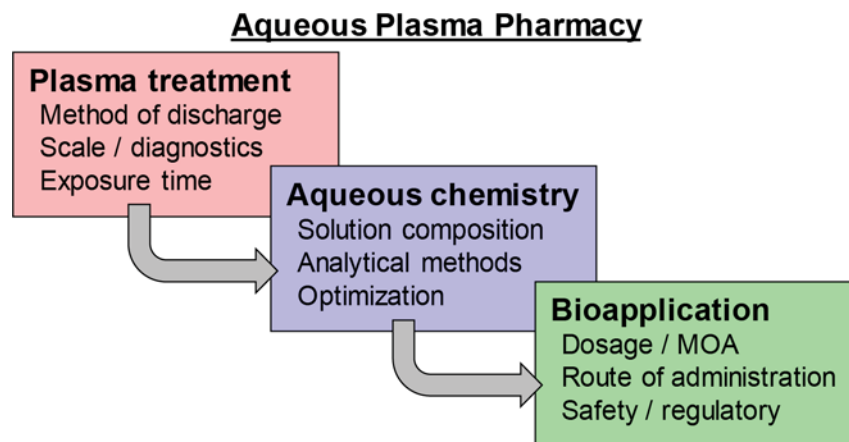
In theory, the use of plasma-activated solutions represents more versatile routes of administration compared to direct plasma plumes with the ability to access internal sites through the bloodstream. Plasma plumes have mostly been suggested for surface disinfection and dermal applications, such as wounds and skin cancer. It has been demonstrated that nonthermal plasma plumes can be operated without dermal safety concerns,<sup>47,198,199</sup> but they are only sufficient to penetrate and thus treat the top 3–5 cellular layers.<sup>75</sup> Direct application of plasma plume species to a prostate tumor site has been suggested via transperineal administration,<sup>19</sup> however, many internal tumors are not so easily accessible as the prostate is rectally. Thus, if stability and composition hurdles can be overcome, it would be more convenient and cost-effective to sell bags of activated solution for administration. Administration of aqueous solutions includes intra-arterial and intraperitoneal routes, among others, where the solution can be delivered at the site of interest, such as a tumor. It is also possible to sell small plasma devices for on-site generation of plasma-prepared bioactive solutions in a systematic and reproducible fashion, where the bioactive solution can be prepared on-site immediately prior to patient administration.

Looking ahead, it is anticipated that the field of APP will extend beyond the applications described above. Plasma-treated solutions have been implicated in electrosurgery applications,<sup>159,200</sup> as well as to enhance the germination, growth rates, and overall nutritional quality of various plants.<sup>201</sup> Many other disease states are known to be related to oxidative stress, such as neurological, cardiovascular, and gastrointestinal disorders in addition to inflammatory and degenerative diseases.<sup>202</sup> Thus, APP may eventually be applied to a variety of indications, such as cardiovascular disease, diabetes, asthma, stroke, arthritis, and renal failure. As an example, a variety of  $\text{NO}_x$  species are involved in NO pathways that are relevant to a variety of vascular applications, such as anti-platelet, antibacterial, vasodilatory, angiogenic, neurotransmitive, and wound-healing mechanisms.<sup>203</sup>

Other potential opportunities include combining plasma-activated solutions with already established small-molecule drugs. For example, the direct plasma treatment of pancreatic cancer cells improved cellular association with subsequent gemcitabine chemotherapeutic treatment;<sup>133</sup> other studies have further indicated combinatorial approaches for direct plasma plumes as anti-cancer therapies.<sup>86</sup> Gold nanoparticles have

been separately investigated for cancer treatment; Cheng et al. demonstrated the effective plasma plume treatment of gold nanoparticles to maximize anticancer efficiency.<sup>204</sup> Other work by Daeschlein et al. indicated that cold plasma therapy can be combined with electrochemotherapy for enhanced therapy for melanoma treatment.<sup>78</sup> Beyond implying combinatorial cancer treatments, work has also suggested that the plasma plume treatment of chemoresistant cancer cells will restore cell responsiveness, thus overcoming chemoresistance issues.<sup>90</sup> These combined and enhanced chemotherapy applications can certainly be applied to plasma-activated solutions.

In conclusion, the field of APP is still in its early stages, but plasma-activated solutions have shown promise for a variety of therapeutic applications, including potential to combat chemoresistant cancers and antibiotic-resistant bacteria. The complex aqueous chemistry yields a multitude of plasma species that perform more effectively than any single oxidant species alone. Furthermore, the transfer of plasma species to the aqueous phase represents a more effective approach than plasma plume exposure alone, and also represents better commercialization potential with the capability to reach internal sites that plasma plumes would not be able to access. More work is required to thoroughly understand the plasma science, chemistry, composition, and mechanism of cellular action for a variety of therapeutic applications (Fig. 6). The work presented to date indicates that this is a very promising field with the ability to address significant widespread unmet clinical needs, such as antibiotic resistance and more effective, selective chemotherapies.



**FIG. 6:** Future work in the field of APP requires more research regarding plasma treatment, aqueous chemistry, and bioapplications.

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