Cold atmospheric plasma delivery for biomedical applications

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Abstract

As the fourth state of matter, plasma's unique properties and interactions with other states of matter offer many promising opportunities for investigation and discovery. In particular, cold atmospheric plasma (CAP), operating at atmospheric pressure and room temperature, has remarkable potential for biomedical applications through various delivery methods. These biomedical applications include sterilization, wound healing, blood coagulation, oral/dental diseases treatment, cancer therapy, and immunotherapy. Effective delivery of plasma constituents is critical to its efficacy for these applications. Therefore, this review presents the key research activities related to CAP delivery (including direct CAP delivery, delivery of plasma-activated media, biomedical device-assisted plasma delivery, and CAP delivery with other therapeutics) and needs for future research. This review will be of great interest for understanding the current state-of-the-art of biomedical applications of plasma medicine and while also giving researchers from a broad range of communities' insight into research efforts that would benefit from their contributions. Such communities include biomedicine, physics, biochemistry, material science, nanotechnology, and medical device manufacturing.

1. Introduction

Plasma (from Ancient Greek πλάσμα), as one of the four fundamental states of matter (i.e., plasma, gas, liquid, and solid), was first described by American chemist Irving Langmuir in 1922.[1] In general terms, plasmas are fully or partly ionized gases. Sufficient energy is added to the gas to strip electrons from atoms, creating a mixture of free electrons, free radicals, neutrals, and positively charged species.[2,3] Free electrons then continue to interact with other species to maintain or increase charged species densities. Most plasmas remain electrically neutral in bulk, which contributes to unique characteristics of plasma and its classification as the fourth state of matter. The properties of plasma (in terms of electronic density or temperature) can change drastically depending on the source and amount of energy supplied. According to Maxwell-Boltzmann thermodynamic equilibrium, plasmas are categorized as either thermal or non-thermal. The category of non-thermal plasma (i.e., not in thermodynamic equilibrium) includes 'cold plasma' since the electron temperature is much higher than those of ions and neutrals.[4] Interest in cold plasma began in earnest with gas discharges in the mid- to late-1970's.[5] These early studies predominantly focused on non-equilibrium plasmas utilizing inert gases such as helium (He) and argon (Ar), as well as reactive gases such as oxygen (O_2), hydrogen (H_2), methane (CH_4), and others. Due to the high cost of generating plasma at below-atmospheric pressures, atmospheric-pressure cold plasma technologies (often called "cold atmospheric plasma" or CAP) have developed rapidly.[1] Such plasma devices have become increasing available and have already shown great promise for a wide range of biomedical applications.

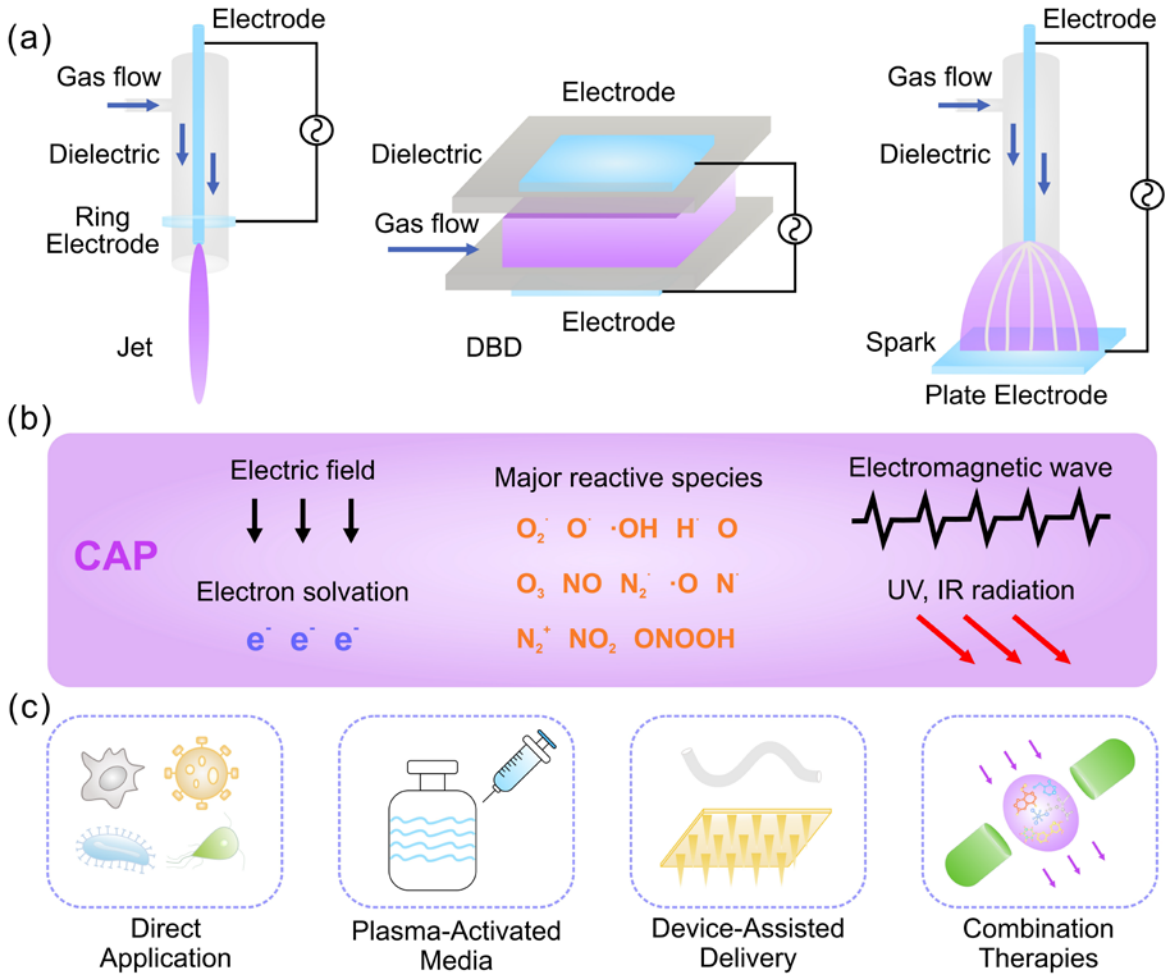

Figure 1. Schematic of cold atmospheric plasma delivery for biomedical applications: (a) typical cold atmospheric plasma configurations for biomedical applications, including plasma jet, dielectric barrier discharges (DBD), and spark, (b) plasma environment containing the plasma generating reactive species, electrons, other ions, emissions, waves, and physical forces, (c) plasma delivery strategies: direct application, device-assisted delivery, plasma-activated media, and combination therapies.

Cold atmospheric plasma (CAP), operating at atmospheric pressure and room temperature, brings together many diverse research fields.[6,7] For medicine, new horizons have been opened for sterilization, blood coagulation, wound healing, skin disinfection, oral/dental disease treatment, cancer therapy, and immunotherapy. In a span of only 25 years, CAP medical applications have made this tremendous leap from initial discovery through the fundamental scientific research stage to clinical applications.[8] In the end, the interdisciplinary research labeled “plasma medicine” has

been formed through innovation and novelty, combining medicine, biology, physics, chemistry, and engineering.[1,9] Plasma medicine brings technological challenges for developing plasma-based medical devices and raises fundamental questions about the interaction mechanisms between plasma and living tissues. The efficacy of CAP in the proposed applications depends on the synergy of free radicals, electric fields, reactive nitrogen species (RNS), reactive oxygen species (ROS), charged particles, and UV photons. The essential roles of ROS and RNS as biologically and therapeutically active agents in a wide variety of intercellular and intracellular processes have become increasingly clear in the past several years.

Researchers are gaining an increased understanding of the acting mechanisms of CAP on cells and tissues.[9-11] CAP therapy relies on the synergistic action of free radicals, the reactive species, UV photons, and charged particles in CAP. In addition, appropriate electromagnetic radiation and electric fields can also benefit CAP therapy.[12-15] It is therefore essential to consider the delivery strategies and mechanisms for CAP for different biomedical applications. Although there have already been a few excellent reviews focused on recent advances, applications, mechanisms, and challenges of CAP,[9,16-22] one that focuses on biomedical delivery strategies for CAP remains elusive. In this review article, we will first highlight the importance of the delivery approaches of CAP for different biomedical applications, then provide a critical assessment of current delivery strategies, and conclude with a future perspective regarding the development and application of CAP delivery (as shown in Figure 1). This particular focus determines the following structure of this review. The Section 2 summaries plasma delivery sources, reactive species generated by plasma source, and their diagnostics. The direct CAP delivery for biomedical applications including sterilization, wound healing, blood coagulation, oral/dental treatment, cancer therapy, and other applications are introduced in Section 3. In Section 4, we demonstrate delivery of plasma-activated media for biomedical applications, such as plasma-activated water, plasma-activated saline solutions, and plasma-activated other media. Biomedical device-assisted plasma delivery for biomedical applications including small tubes employed to deliver plasma, plasma delivery through micro-sized tube, and CAP delivery through patch, are discussed in Section 5. Section 6 highlights plasma delivery with other therapeutics containing nanoparticles and immunotherapy. Section 7 summaries conclusions, challenges, and future opportunities for studies of plasma delivery for biomedical applications. This article aims to facilitate researchers from

fields, including material science, biomedicine, physics, biochemistry, nanotechnology, and medical device manufacturing, to grow their body of knowledge and expand their understanding of the physical and biochemical pathways whereby CAP is delivered to biological subjects.

2. Cold atmospheric plasma delivery

2.1 Plasma sources for delivery

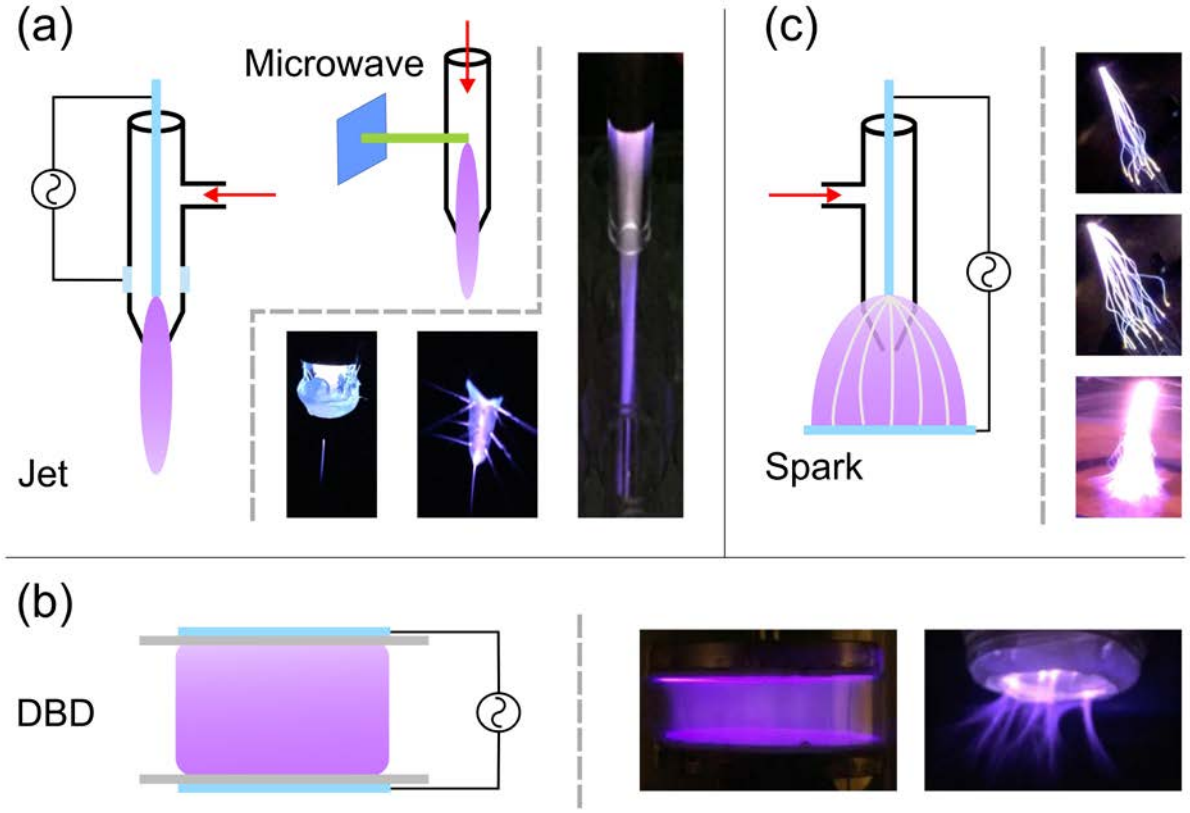

Figure 2. Typical CAP system configurations for plasma delivery with discharge images: (a) plasma jet, (b) dielectric barrier discharges (DBD), and (c) corona discharges.

CAP is a unique type of plasma, which includes neutral atoms and molecules, electrons, radicals, electric fields, excited states, UV photons, and ions. It can be obtained by a range of different electrical discharges such as glow discharge (plasma jet, plasma needle, plasma pencil, and dielectric barrier discharge (DBD)), and corona discharge.[23] There are many categories used to classify CAP sources, including plasma discharge mode, interaction characteristics with objects, and excitation frequency. Classification according to electrical configuration and plasma/object interaction characteristics provides the most general distinction, with various kinds of CAP developed in each. As shown in Figure 2, plasma jet, DBD, and corona discharge are common sources for CAP generated at atmospheric pressure. Figure 2 also shows images of plasma discharge for each configuration. Plasma generation between two electrodes, as used in most CAP

devices, does not occur spontaneously.[24] Electric field intensity and mean free path for electrons define the basic properties that must be within the proper ranges to initiate the discharge, with the latter dependent on the carrier gas density and pressure as well as the size of the gap. With the restriction of operating at atmospheric pressure and fixed gap size, the main variable is then the electric field intensity, which is generally increased through applied voltage and current.

As a glow discharge, a plasma jet is the extension of an active discharge or decay column from the location of the generation.[25] Typical plasma jets are configured in an annular structure with the gas flow through the central channel. This flow produces the "jet" for which these devices are named.[26-29] Plasma jets can also be generated from different configurations, including modified DBD and arc discharges.[9] Plasma jets are often more appropriate for targeted modification of three-dimensional or in-situ structures, and unlike most other forms of plasma generation, plasma jets do not require the subject to be passed between the electrodes in order to interact with the discharge.[30] Different configurations, flow rates, and electrical characteristics can be implemented to change the nature of the jet. For large surface/object treatment, multiple plasma jets or plasma jet arrays have been invented.[31-35] Several smaller plasma jets for plasma delivery have been referred to plasma "pens", "pencils", or plasma needles, and have been used for finely controlled medical applications. Separately, DBD devices can be designed in either planar or tubular configurations depending on the delivery purpose: planar orientations allow for a significantly larger area for surface or material modification as well as uniformity in application, while tubular DBDs are often used when the targets are gases themselves such as for various biomedical applications.

Corona discharges are generated by creating an electrical field strong enough to ionize the gas surrounding an electrode but not strong enough to generate arcing or electrical breakdown.[36] They can be generated through a wide range of direct current (DC) and alternating current (AC) potentials, are generally dependent upon the ionization of carrier gas, and are characterized as positive or negative coronas depending on the electrode at which the discharge forms. Most naturally occurring atmospheric plasmas are generated by corona discharge, with lightning being perhaps the most familiar.[37] Corona discharges most commonly appear as streamer or spark discharges for industrial applications, especially when etching or other surface modifications of

the target surface are desired.[38] Glow discharges that do not include a streamer or spark may be used in circumstances where high electric current or sudden sparking may damage the subject, such as when delivering with living tissues. Pulsed discharges can likewise be used to generated high-power fields without leading to arcing or sparks. When glow discharges are preferred, inert elemental gases are more commonly used, as the characteristics of molecular gases tend to lead to increased streamer formation. Arc discharge is characterized by lower voltage and higher current than glow discharge, while glow discharge depends on the electrons thermionic emission of the electrodes supporting the arc, which is related to the thermal, chemical, and electrical phenomena in the region.[39] Arc discharge can be applied for ultra-fine particle synthesis, plasma spraying, cutting (tissue), metal welding, biogas production, waste treatment, etc.[40] This review paper briefly introduces the glow discharge for biomedical applications and its delivery.

Electrosprays and plasma sprays, as an alternative to plasma and plasma treated solutions delivery, bring a wide range of potential biomedical applications. Jaworek *et al.* summarized the physical processes and phenomena of the combination of electrosprays and CAP.[41] Plasma sprays can reduce the size of droplets and increase the surface area to volume ratio, thus enhancing the transfer of active substances produced by plasma to water. The plasma sprays prepared by this method have strong antibacterial effects. The combination of plasma and aerosols is used in situations where direct contact needs to be avoided.[42] The development of plasma sprays has also led to a deeper understanding of extremely complex chemical reactions. In addition, Stancampiano *et al.* showed that plasma sprays create new opportunities in enhancing the transfer of activation energy from plasma to liquid,[43] controlling reactivity in liquid, in-flight production and on-demand delivery of design micro/nanomaterials related to clusters and/or liquid evaporation, and delivery of short-lived species.

2.2 Plasma-generated reactive species for delivery

With thermal effects minimized due to the lower bulk temperatures, CAP discharges mainly rely on the high-energy reactions that generate the discharge itself and continue to occur throughout the plume and on interaction with the subject, with the plasma-subject interaction playing a significant role in the generation of species in many applications.[44-49] Roughly, three distinct groupings can be defined in plasma chemistry: the initiation and maintenance of the discharge, the

reactions occurring within the discharge between the carrier gas and any other gases introduced, and the reactions that occur in contact with the subject or at other surfaces or interfaces.[50] Once the proper intensity is reached, a single free electron generated either through deliberate action or stochastically from environmental conditions and background radiation can then initiate a Townsend Avalanche. The avalanche occurs when the free electron has sufficient energy to collide with multiple neutrals within the carrier gas, each collision causing an ionization event and releasing additional electrons. The nature of the resulting discharge varies with increases in applied current to the electric field: immediately prior to breakdown voltage, a dark or invisible corona forms, which then collapses at breakdown to a glow discharge.[51] Further increases in current drive the discharge to streamer and arc formation, with additional increases resulting in a transition to a thermal arc state. Once the avalanche is initiated, the bulk of the carrier gas remains neutral while only a small portion becomes ionized at any given moment, generally most abundant in the vicinity of the anode. As such, the primary reactions are electron-neutral interactions resulting in the generation of standard ions of the carrier gas.

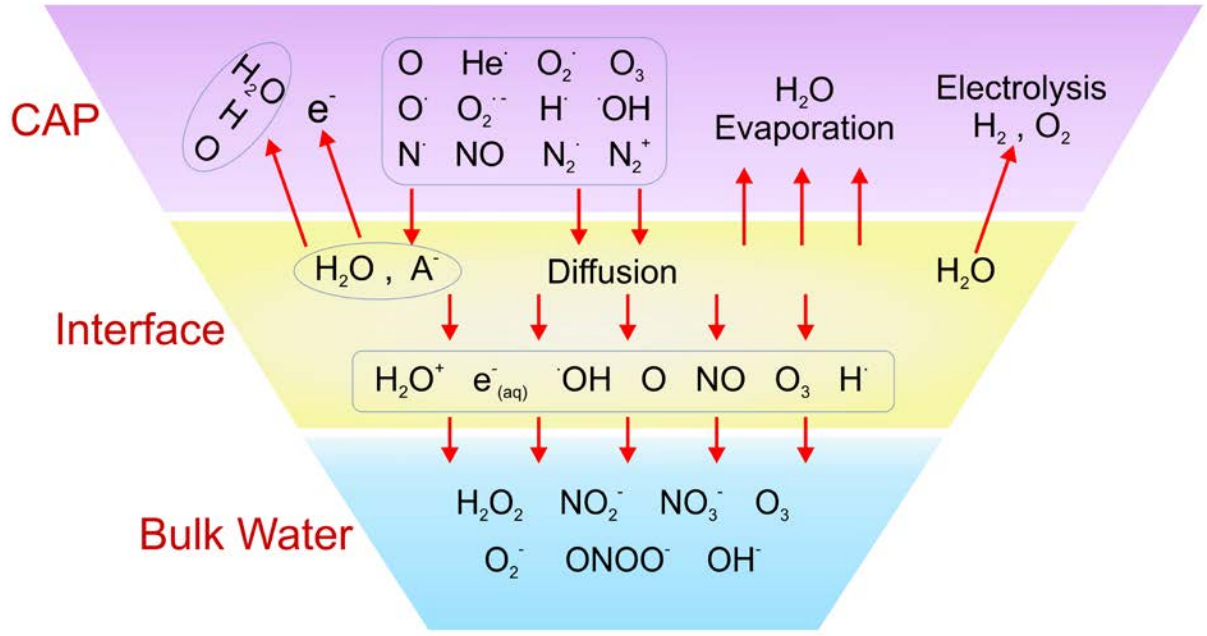

Figure 3. Generation, transport, and interactions of reactive species delivered to cells/tissues/liquid.

Once the discharge is formed, equilibrium is reached as ions escape the discharge to either re-merge with free electrons or interact with the boundaries of the gap. If these boundaries are metal electrodes, collisions between positive ions and these metal boundaries result in the release of secondary electrons into the gap. Further, with sufficient ions present in the discharge itself, electron-ion interactions can begin to occur; these reactions can have numerous results, including excitation of electrons, ions, and neutrals, as well as secondary ionization and further reactions. When the discharge is allowed to mix with other gases, such as standard air, these reactions can rapidly become extremely complex; hundreds of specific reactions have been documented in open-air cold atmospheric plasma discharges through spectroscopy and other methods, many leading to generation of reactive oxygen and nitrogen species in particular (ROS and RNS, respectively). While difficult to model or control, these reactive species are responsible for many of the advantageous uses of CAPs, especially when used with biological tissues. Figure 3 shows the generation, transport, and interactions of reactive species delivered to cells/tissues/liquid. Some of the more critical species are described below.

The hydroxyl radical (•OH) is often considered as the main driver of biological effects that results from CAP treatment.[52] •OH has bactericidal effects, as it participates in the destruction of cell membranes and interferes with DNA and other intracellular materials.[53] Hydroxyl radicals have also been verified as a major component of nitric oxide neutralization in certain circumstances.[54] They are primarily produced within the plasma jet itself when water vapor is present within the feed gases and are most effective when exposed immediately to the subject due to their relatively short lifespans. The aeration of a liquid medium with the plasma discharge can increase exposure of a liquid-born subject to hydroxyl radicals.[55] In addition, the formation of hydrogen peroxide (H_2O_2) seems to be primarily driven by humidity in the feed gas, with a minor amount produced by interaction of the plasma jet with any ambient vapor arising from the subject or surrounding atmosphere.[52] Although multiple reaction pathway exists, the primary reaction driving H_2O_2 formation is a two-step process mediated by the production of •OH. As a result, reduction of hydroxyl radicals by any means including the presence of various scavengers likewise leads to a decline in H_2O_2 production at the sample/plasma interface.[56] Further, the increased presence of nitrogen in the feed gas leads to a decrease in H_2O_2 production as measured in plasma-activated

water, with speculation that the increased nitrogen presence contributes to production of peroxynitrate and reduces the free electrons available for hydrogen peroxide formation.[57]

As a well-known oxidant and precursor to hydrogen peroxide formation, superoxide (O_2^-) is mostly neutralized within cells by superoxide dismutase.[58] O_2^- is also short-lived in aqueous solutions and has limited ability to bypass cell membranes, leading to most oxidation driven by O_2^- occurring at the surface of the cell.[59] For example, the charged and energized superoxide anion radical is implicated in the breaking of seed dormancy and thus speeding germination.[60] On the other hand, the main production mechanism of atomic oxygen (O) is electron-induced, which plays a significant role for cells.[61] Singlet delta oxygen ($O_2(^1\Delta g)$) is an important oxidant in cells and tissues, which could be neutralized in cells by the enzyme superoxide dismutase (SOD), forcing cells to rely instead on low-molecular-mass compounds like carotenoids for antioxidant defense.[62] One study found that $O_2(^1\Delta g)$) production was limited to gaseous interaction within the plasma jet, with an inverse relationship between the production of $O_2(^1\Delta g)$) and the proximity of the jet to an aqueous surface interface.[63] Due to its excited state, $O_2(^1\Delta g)$) is highly oxidative, leading to a tendency to initiate lipid oxidation.[53] It has more thoroughly been implicated in cancer tumor cell death through a self-amplifying catalase inactivation within tumor cells.[64]

The effects of ozone (O_3) in general are well-documented. Ozone generated *via* non-thermal plasma has been used for various purposes. For example, elemental mercury was shown to be oxidized primarily by O_3 when exposed in simulated flue gases processed through a DBD.[65] Hydroperoxyl radical (HO_2^*) is a compound generally produced in plasmas through the interaction of free hydrogen with molecular oxygen, though it can also be produced through a secondary reaction of hydroxide with hydrogen peroxide.[66] Thus, for reasonable HO_2^* production, both molecular oxygen and water vapor should be present in the feed gas.

Nitric oxide (NO) and other NO_x molecules are also produced within the plasma discharge when both nitrogen and oxygen (either in diatomic gas form or bound in other compounds) are present. In CAPs, the primary reaction chain for NO production is through nitrous oxide formation (either *via* a long chain of NO_x reactions and interactions or through mediated oxygen and nitrogen gas interactions) and disassociation into nitric oxide in the presence of free oxygen or ozone.[67] While

generally considered as an atmospheric pollutant, nitric oxide itself has been shown to have specific properties in medical applications that may be desired. For example, concentrations of NO in plasma-activated water have been shown to both boost macrophage activation and effectiveness as well as induce apoptosis in cancer tumor cells.[68,69] NO has also been shown to affect yeast growth, with low NO concentration plasma-activated water boosting growth while high-NO-concentration water inhibited growth and even led to death.[70] An abundance of other reactive species are generated in CAP, and we will discuss them as relevant in the following biomedical applications.

Concentration of both ROS and RNS in CAP should be paid much attention to the balance between physiological beneficial regulation and pathological harmfulness.[71] •OH, a type of ROS, stimulated cyclic guanosine monophosphate (cGMP) formation, showing that cells can not only coexist with free radical production atmosphere but are capable of utilizing these substances for cellular, physiological use.[72] Similarly, NO, a type of RNS, can generate peroxynitrite for further oxidation stress and can mediate cellular toxicity,[73] and the role of NO as a crucial physiological process regulator in blood vessel modulation cannot be ignored.[74] The role of ROS in cellular protection mechanisms against excessive tissue dysfunction have been mentioned. High-dose ROS induce mitochondrial structural damage *via* MPT pore opening characterized by mitochondrial swelling, a process called mitoptosis, which can subsequently induce a series of antioxidant defenses to avoid further oxidation.[75,76] Similarly, for low-dose ROS/RNS, autophagy can be seen as a type of mechanism beyond cell death as a risk-remover to benefit cell survival. Based on mitoptosis, dysfunctional and damaged mitochondria are further removed by autophagy mechanism to avoid further apoptosis for normal cells,[77] the process of which was proved by living hepatocyte experiments.[78] Results on ROS/RNS dose-related experiments were also carried on in 3D skin, showing that low-dose CAP treatment presented good cell compatibility, suggesting the beneficial effect of CAP low-concentration ROS/RNS delivery.[79] Thus, CAP treatment time should be thoroughly investigated, and low-dose ROS/RNS CAP device establishment is suggested.

2.3 Plasma diagnostics

Given the complexity of interactions and reactions within plasmas in general, characterization and analysis of CAP discharges is largely approached empirically rather than theoretically.[80] This is a known weakness in the field: while significant advancement is taking place on the application of cold atmospheric plasma across a large range of disciplines, the underlying theoretical framework for these plasmas is only modestly understood. Although generalities can be assumed in many cases, diagnostics of a given plasma discharge and device are done at a more basic level, with each individual implementation requiring distinct analysis to determine the specific products generated by the device; such diagnostics are done largely through electrical analysis, spectrometry, spectroscopy, intensified charge-coupled device (ICCD) imaging, and schlieren visualization. Traditional imaging is limited to characterizing hydrodynamic phenomena. Schlieren visualization can often qualitatively and effectively characterize the hydrodynamics of plasma jets, helping overcome the challenges of hydrodynamic instability and uncharacterized turbulent regions at the end of the jet. By changing the key parameters, such as peak voltage (PV), pulse repetition frequency (PRF), and mass flow rate (Q), the effect of high-speed schlieren imaging on the hydrodynamics of plasma jets can be studied. Boselli *et al.* performed hydrodynamic characterization of high-voltage pulse-driven plasma jets by schlieren high-speed imaging.[81] Using a combination of high-speed imaging cameras and schlieren visualization, they visualized the time-evolving fluctuations produced by the plasma jet as it hits different substrates. Robert *et al.* demonstrated that the hydrodynamic effects of plasma on noble gas flow can be visualized using ICCD imaging and schlieren.[82] Key factors affecting gas flow include the geometry of the plasma within the tube prior to ejection, the presence of a metal target, and the pulse repetition frequency. The results show that the plasma plume acts in the opposite direction at moderate gas flow, the plasma plume is stable and unexpanded in laminar flow at higher gas flow, and conversely, the plasma plume is generated in laminar flow caused by plasma ignition.

Gazeli *et al.* investigated the temporal and spatial distribution of the absolute density of Ar when an unground glass surface is impacted by a microplasma jet.[83] The absolute density of Ar was quantified by TDLAS combined with ICCD imaging and emission spectrum. Experiments showed that the maximum density had a strong dependence on gas velocity and spacing. The metastable density of Ar near the surface of the glass plate may be high enough to induce molecular desorption without damaging the surface. Darny *et al.* used schlieren imaging to study helium flow correction

caused by plasma jet at low helium flow rates.[48] It was shown that the channel formed by applying a negative pulse emits an ionization wave, which makes the plasma plume generated by the positive voltage pulse stable enough to expand downstream. Jiang *et al.* studied the interaction between discharge mode and airflow in an atmospheric pressure plasma jet.[84] Plasma flow was filmed using schlieren visualization. The experimental results show that the gas is always driven forward by the large voltage generated by a single active electrode. The DBD mode, however, creates a glow discharge between electrodes that creates turbulence. In addition, a combination of laser-induced fluorescence (LIF), particle image velocimetry (PIV), and computational modeling methods can also be used to quantify the hydrodynamic effects of air entrainment in plasma jets. Morabit *et al.* used LIF, PIV, and numerical simulations to explore the effects of plasma plumes in the air.[85] It turns out that the velocity of the helium flow is independent of the plasma, but the plasma induces perturbations in the shear layer that emanates downstream and causes turbulence.

As the electric field driving the ionization of the carrier gas is the most critical aspect of the plasma device, characterization of the field itself is one of the important goals of plasma diagnostics.[86] This is done through two major avenues: the properties of the device itself as an electric circuit, and properties of the realized field and resulting discharge between the electrodes.[87] The former is typically trivial to determine, so that commonly reported values in plasma experiments include applied voltage, current, and (for AC devices) the frequency of operation. Measurement of the properties of the resulting field is somewhat more complicated, as the introduction of any probe or device into or even near the discharge can alter the nature of the discharge in ways that are not always predictable. The underlying complexities involved in the experimental measurement of plasmas have been described within the literature through both the precision and specificity with which measurements are defined and executed in various studies as well as the dialog within the community on the proper ways to conduct and interpret such measurements.[31,88-92] With care, however, reasonable approximations of field characteristics can be determined. The most critical values for measurement are the electron temperature (T_e) and charge density (n) between the electrodes, as these values can then be used to determine other critical plasma characteristics.[93] Additionally, the strength of the realized potential between the electrodes as well as the current flowing through the discharge can be measured.

In addition to the characteristics of the plasma itself, understanding of the reactions happening within the discharge and between the discharge or its effluents and the subject also forms a critical role in plasma diagnostics.[94] While modeling can be informative for simplified experiments, such as when using a pure elemental carrier gas, exposure to real or synthetic air mixtures as well as subjects with complex chemical composition introduce an extreme amount of complexity to the reaction mix. It is therefore generally more practical and accurate to measure and then determine the nature of reactions happening within the discharge or at the surface by examining the products of the reactions described above. Mass spectrometry can provide a detailed analysis of the composition of a plume through sampling of the discharge effluents. Neutral and charged species must often be analyzed separately due to the nature of mass spectrometry, because the possible disequilibrium state of discharge can lead to some discrepancies in the results.[95] Complicating matters, shorter-lived reactive species may not persist long enough to be measured, and thus their presence in the discharge may be more difficult to ascertain. Further, as stated with other diagnostic methods, sampling of the discharge may change the nature of the discharge itself, and therefore mass spectrometry is most often used either with plasma jets or in pre-experimental analysis where such samples can be obtained without altering the experimental results.

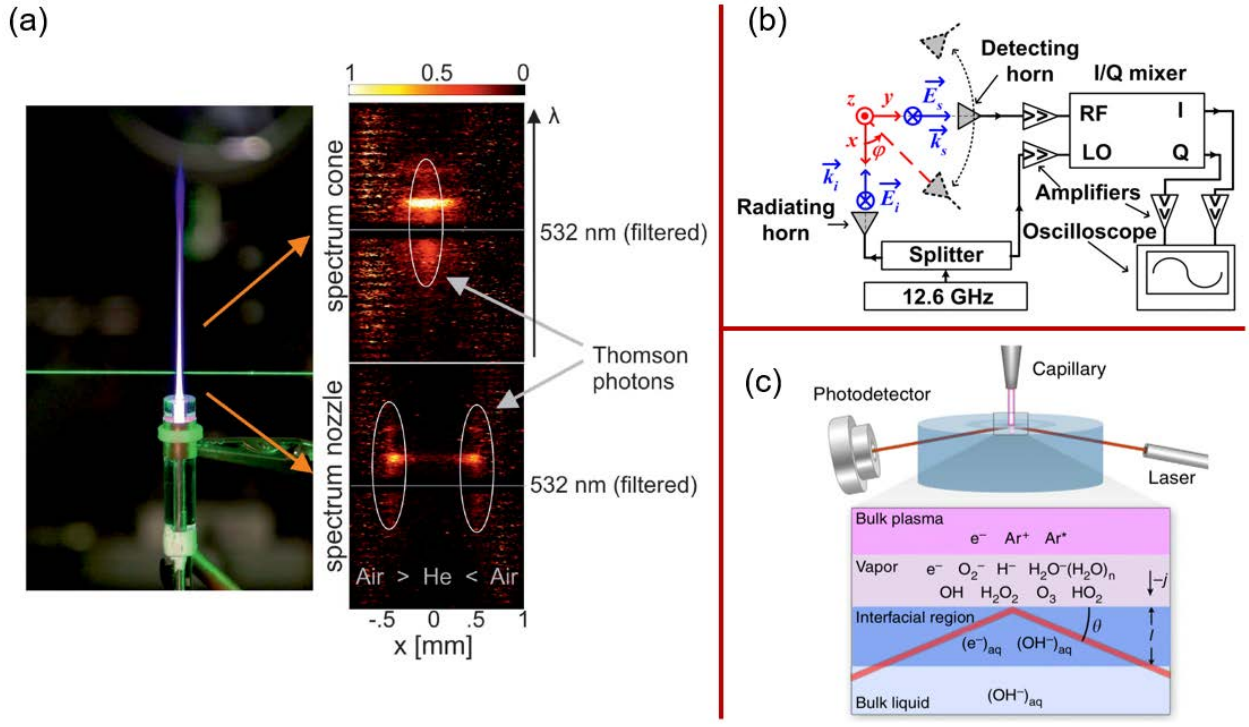

Figure 4. CAP diagnostics: (a) Thomson scattering technology determined electron properties of the atmospheric He plasma jet. Shown are also two representative laser scattering spectra from different axial

positions (top: z = 11 mm; bottom: z = 2 mm). Reproduced from reference.[96] Copyright 2014, IOP Publishing Ltd. (b) Schematics of Rayleigh microwave scattering (RMS) experimental setup. Reproduced with permission.[97] Copyright 2010, American Institute of Physics. (c) Schematic of experimental apparatus for optical detection of solvated electrons using a "total internal reflection" configuration. Reproduced under the terms of the CC BY license.[98] Copyright 2015, Nature Publishing Group.

Emission spectrometry is another approach to the analysis of discharge composition. Optical emission spectrometry (OES) is a commonly used diagnostic method for all forms of CAP, as it is entirely passive in its mechanism and can be used simultaneously with active experimentation and subject treatment. Additionally, using spectra ratios between known standard species can provide a secondary method for determining the electron excitation temperature within the discharge. Although fluorescence and emission measurements are generally non-invasive with high time and spatial resolution, the necessity of several assumptions regarding the model reduces their accuracy. Alternatively, scattering techniques that leverage elastic scattering from electrons (Thomson) or heavy particles (Rayleigh) or inelastic scattering from molecules (Raman) are particularly useful. Figure 4a shows the Thomson scattering technology determining electron properties of an atmospheric He plasma jet.[96] In the surrounding air, the strong Raman scattering of the laser beam has been subtracted. The electron density in the vicinity of the plasma jet nozzle has a maximum value of 5×10^{18} m^{-3}, and mean energies are up to 2.5 eV. The Rayleigh microwave scattering (RMS) was first proposed by Schneider in 2005 for measuring electron density in plasmas with structures smaller than microwave wavelength. It was then used to determine laser-induced avalanche ionization and resonance-enhanced multiphone ionization in air and argon plasma, respectively.[97,99] The schematic of the RMS experimental setup is shown in Figure 4b. For surface analysis, energy dispersive x-ray spectroscopy performed before and after treatment can identify specific chemical and molecular changes to the subject resulting from plasma exposure. Especially when used with sputtering or etching experiments, this can be an effective way to identify species present in the discharge that become impregnated on or just below the surface of the material. Further, specific reactions at the surface, such as oxidation or polymerization, may be driven primarily by the presence of specific reactive species or ions within the discharge; as such, evidence of those reactions can be used as indirect confirmation of presence for those species within the discharge. Figure 4c shows internal reflection as used to measure the strong red absorbance (λ_{max}~700 nm) of solvated electrons generated by plasma. Runbach *et al*. employed

individual diode lasers working in tandem through a 163 mM sodium perchlorate solution to determine the absorption spectrum at the plasma-solution interface. Reactions with various electron scavengers including H_2O_2, H^+, NO_2^- and NO_3^- show that the kinetics of CAP were similar, but not exactly the same as, the kinetics of solvated electrons formed in a large amount of bulk water by radiolysis.[98] Other diagnostic tools can also be of value depending on the nature of the specific experiment. While CAP is inherently expected to show little thermal variation from ambient surroundings or a subject, thermal imaging can provide additional confirmation of this. Visual tools, such as high-speed cameras, visual or electron microscopes, and ICCD cameras can provide additional information about the plasma and its properties.

3. Direct CAP delivery for biomedical applications

CAP can produce a chemically rich environment at close to atmospheric pressure and room temperature, a unique condition that can deliver highly reactive species in a beneficial and non-destructive manner.[17] Emerging as a novel technology, It can be applied to plant, animal and human tissues without harm. Due to distinctive biological effects, CAP has potential as new plasma-enabled healthcare solutions with applications in sterilization,[8] wound healing,[100] blood coagulation,[101] oral and dental treatment,[102] cancer therapy,[103] and others.[104] The relevant works and recent developments of direct plasma delivery for these applications are discussed in this section.

3.1 Sterilization

Although the traditional sterilization methods, such as γ-ray, electron beam, humidified ethylene oxide gas, heat-based, and steam sterilization, are extensively used, it is urgent to develop innovative and improved technologies for disinfection, pasteurization, and sterilization against resistant bacteria.[105] For example, heat-resistance microorganisms which induce Creutzfeldt-Jacob disease (mad cow disease), appear due to long-term and extensive utilization of heat-based methods.[106] Plasma sterilization is an emerging non-thermal technology for inactivating bacteria, germs, viruses, and spores that is receiving great attention in biomedical applications.[107] Moreover, due to the limitations of the above-mentioned traditional sterilization methods, such as toxicity, weak penetration, rescued material service life, or altered material properties, it is difficult to ensure that such methods can achieve a suitable level of sterility while maintaining the compatibility of functions and materials.[105] The direct attack of the microbial structure by the plasma-generated reactive species to keep the number of germicidal spores constant allows gas plasma to show great potential for sterilization. CAP can sterilize human tissue with minimal or no damage and kill bacteria or spores in a variety of food materials without compromising the main physical-chemical characteristics of the food.[108-110] The plasma inactivation efficacy depends on system and process variables including gas composition, power input, and the duration and mode of exposure as well as intrinsic features of the target (microbial cells), primarily delivery dosage and cell types. Figure 5 shows survival rates of viruses treated by plasma and their potential mechanisms. The brush-shaped plasma plume, employing He as the feeding gas at 10 kV discharge voltage and 11 kHz frequency, is shown in Figure 5a (upper). This plasma plume employing 5/95

O_2/He as feeding gas was applied to Candida albicans spore, and the results indicated high efficiency for the plasma inactivation of the resistant Candida albicans spores (scanning electron microscope (SEM) results in Figure 5a (lower)).

Feeding gas type plays an important role on the type and range of reactive species generated with an expected significant effect on micro-organisms.[111,112] Figure 5b exhibits the survival curves of Enterococcus faecalis bacteria exposed to a plasma brush employing He with the addition of different amounts of O_2. 2.5% O_2 addition induced a faster response for a complete kill within a 60 s treatment, while the pure He plasma brush needed 120 s to kill all bacteria. The addition of O_2 decreases He molecules' emission rates, which leads to enhancing the concentration of ROS.[113] However, O_2, an electronegative gas, more easily absorbs the electrons in plasma, which results in significantly reducing electron density of He/O_2 plasma at higher O_2 fractions.[114] Figure 5c displays the survival rates of E. coli treated by air, N_2, and O_2 plasma with 30 s duration. The N_2 plasma slightly decreases the survival rate of E. coli while the air and O_2 plasmas rapidly decrease the survival rate to 1%-4%. The charge accumulation caused electrostatic forces on the outer membrane of bacterial cells, which overcame the tensile strength of the membrane and resulted in its rupture.[115] The N_2 plasmas did not play an important role in the sterilization process due to its high survival rate at 83%. Lu *et al*. also indicated N_2 plasma resulted in a high survival rate, which is consistent with this result.[116] The O_2 and air plasma are excellent sources of ROS, such as O, • OH, and O_3, which are identified as a major contributor in sterilization.[117,118]

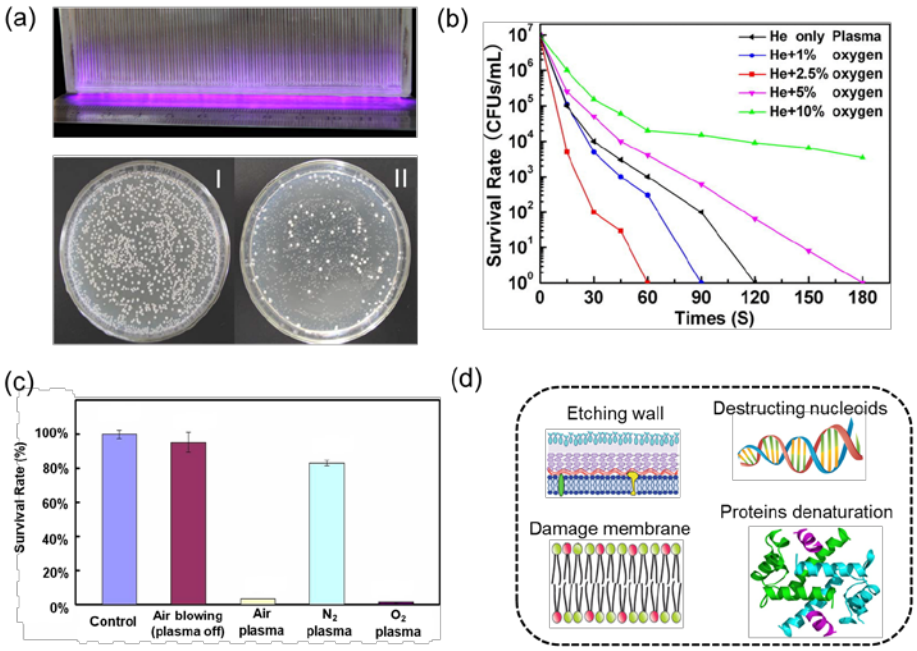

Figure 5. Plasma delivery for sterilization: (a) Photograph of the brush-shape plasma plume (upper) produced employing He as feeding gas with 110 mm length and 10 mm width, and SEM images (lower) of the Candida albicans spore without plasma treatment (I) and with 5/95 O_2/He plasma treatment (II). Reproduced from reference.[119] Copyright 2012, Institute of Electrical and Electronics Engineers. (b) Survival curves of Enterococcus faecalis bacteria treated by He plasma brush with different O_2 additions. Reproduced with permission.[120] Copyright 2012, American Institute of Physics. (c) Survival rates of E. Coli treated by air, N_2, and O_2 plasma with 30 s duration. Reproduced with permission.[121] Copyright 2011, American Institute of Physics. (d) Potential mechanisms of plasma action on virus resulting into loss of functionality and sterilization, including etching virus walls, damaging virus membranes, destruction of genetic materials, and protein/enzyme denaturation.

CAP has exhibited its great potential against different types of bacteria, and its antimicrobial efficacy depends on plasma delivery dosage (reactive species) and bacteria types. It is of note that CAP treatment is as effective against nonresistant bacteria as for resistant bacteria and viruses. Maho *et al.* tested a CAP multi-jet device that induced inactivation for several strains of bacteria,[32] including single bacteria strains (*Pseudomonas aeruginosa, Staphylococcus aureus*), and even drug-resistant strains (*S. aureus, P. aeruginosa & Escherichia Coli strains*). Similarly, the experiment by Sakudo *et al*. showed ampicillin-resistant, transformed *E coli DH5alpha* was also sensitive towards 2-minute treatment with a DBD plasma, characterized by bacterial DNA damage and lipopolysaccharide (LPS) loss.[122] Decrease in the chloramphenicol acetyltransferase (CAT)

expression post CAP treatment in such types of *E coli* bacteria from both CAT assay and enzyme-linked immunosorbent assay (ELISA) assay provided further support for the stated result, as the CAT gene is responsible for detoxifying the antibiotic chloramphenicol to establish drug-resistance. CAP can even be applied for airborne indoor transmission mitigation.[123] Antibacterial activity of bioaerosol on S. epidermidis, a type of common skin and mucosal microbiota, as well as degradation of SARS-CoV-2 RNA, was observed after CAP treatment with a short residence time (less than 0.2 seconds).

Reactive species generated through various collisional pathways play a dominant role in plasma inactivation through oxidative stress.[124] Antioxidants, such as water-soluble α-tocopherol and ascorbic acid, stands out as the best in scavenging ROS and protect bacteria from oxidation effects.[125] However, these antioxidants failed to protect and revive bacteria after exposure to plasma, which indicates plasma inactivation may be irreversible. Some literature reports no clear differences in plasma treatment related to cell wall structure, while others indicate plasma treatment induce different bacterial sensitivity based on cell wall structures.[126-128] Computer simulations of damaged bacterial cell walls induced by CAP were developed by Maksudbek *et al*., and indicated that ROS in plasma broke important structure bonds, such as C-O, C-C, and C-N.[129,130] Four basic potential mechanisms of plasma inactivation contribute to bacterial cell death (as shown in Figure 5d), including etching cell walls, damaging cell membranes, destruction of genetic material, and protein/enzyme denaturation. Bacterial cell dysfunctionality and wall leakage induced by CAP were the synergetic effect between generated reactive species and the microorganism itself.[131,132] Massive reactive species were transported through the membrane, dysregulating membrane lipids *via* oxidative damage; charged particles accumulating on the bacteria's surface also destroyed the cell membrane by electrostatic disruption.[133,134]

ROS can cause oxidation of unsaturated fatty acid peroxides, nucleic acids, and amino acids *via* interaction with membrane lipids, resulting in changes in the membranes' function.[135] Joshi *et al*. demonstrated that plasma-generated ROS induced oxidative stress that affected t cell membranes and led to DNA-associated changes.[136] ROS inducing lipid peroxidation yields products that react with proteins and DNA in biological membrane systems, in turn causing oxidative modifications.[137] Peroxidation of lipids further gives rise to reactive species, which decompose

through catalytic transition with metal ions or chemical degradation to generate hydroxyl radicals, oxy radicals, and reactive aldehydes.[138] Then, reactive aldehydes give rise to DNA adducts that extensively damage DNA and lead to bacteria death.[139] Continuous oxidative damage of DNA is a significant contributor to plasma inactivation. ROS delivery affects the nucleoid to inhibit cell replication *via* forming thymine dimers and strand breaks.[140] In protein/enzyme denaturation, amino acids are modified by ROS so that the secondary structure changes, which leads to loss of recognition and catalytic function.[141] Bacterial inactivation by plasma treatment is a complex process, and its action mechanism is a subject of interest that is still not totally understood.

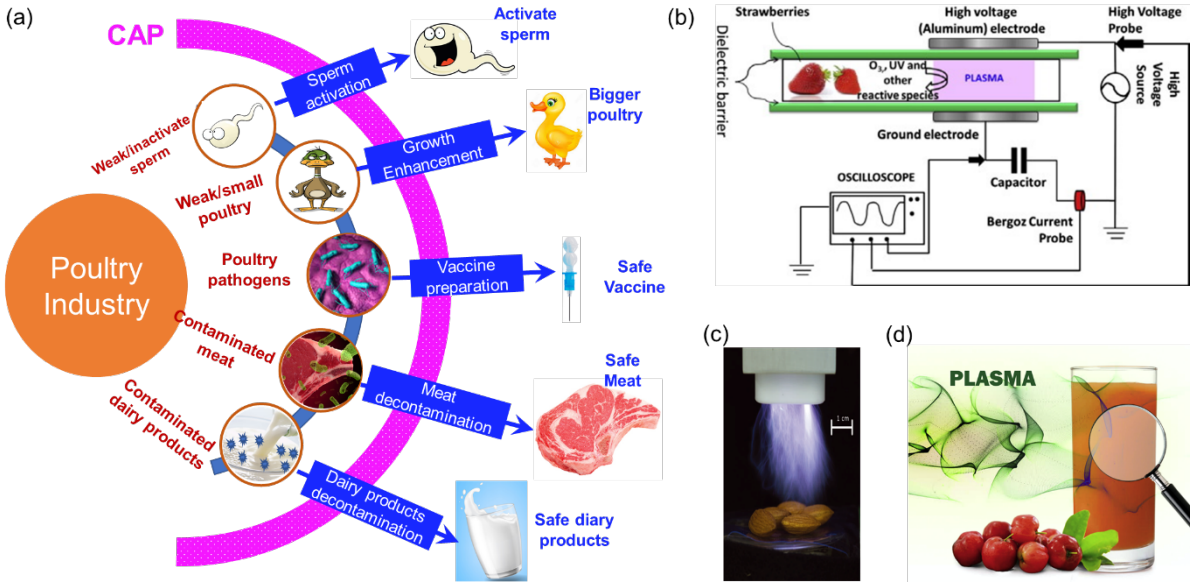

Figure 6. Plasma delivery for food sterilization and quality. (a) CAP for the poultry industry.[142-144] (b) Strawberries treated with CAP, at a 60 kV pulsed at 50 Hz across a 40 mm electrode gap, reduced the background microflora (aerobic mesophillic bacteria, yeast and mould) by 2 log10. Reproduced with permission.[145] Copyright 2014, Elsevier. (c) Plasma reducing Salmonella and Escherichia coli O157:H7 on almonds using ambient pressure gases. Reproduced with permission.[146] Copyright 2012, Wiley-VCH. (d) CAP treatment could be adjusted to have a positive influence on vitamin A, carotenoids and color of acerola juice. Reproduced with permission.[147] Copyright 2019, Elsevier.

Food safety, relating to processing, packaging, and storage, is one of the major health concerns worldwide due to the occurrence of food contaminants including allergens, histamine, polycyclic aromatic hydrocarbons, mycotoxin, and pesticide.[148-151] Food contaminant is a huge challenge for the food industry because of the inefficiency of conventional decontamination techniques. CAP as

an emerging technique has attracted notable attention due to its sterilization of food and degradation of food contaminants. CAP has been shown to efficiently degrade food allergens (e.g. b-conglycinin, tropomyosin, trypsin inhibitor, Kunitz type trypsin inhibitor, and glycinin) and pesticides (e.g. cypermethrin, paraoxon, chlorpyrifos, parathion, omethoate, fludioxonil, dichlorvos, azoxystrobin, malathion, and cyprodinil).[152] These degradations are caused by ROS and RNS in the CAP that break the chemical bonds of the food allergens and pesticides. Figure 6a shows that CAP has a great potential to decontaminate eggs/chicken and enhance the production yield in chicken farms *via* vaccine production, sperm activation, and growth enhancement. Many previously conducted microbiological studies confirm the significant decontamination effects of CAP against poultry product-associated spoilage and pathogenic bacteria.[153-158] They also indicated that different bacteria have various survival rates under the same plasma delivery conditions.

Figure 6b shows CAP treatment of strawberries for microbial inactivation. After plasma treatment, the total yeasts/mold and mesophilic counts of strawberries were significantly reduced by 95% and 85%, respectively, without adversely affecting the firmness and color or inducing significant physiological stress. Plasma in air is considered to be comprised of over 75 species and around 500 reactions, which inactivate the bacteria before reverting to their stable or original states.[159-161] Other researchers also demonstrated the ability of CAP to reduce inoculated microbial populations on fresh produce surfaces of fruits and vegetables (e.g. apples, cantaloupe, potato, and lettuce).[135,162,163] Escherichia coli O157:H7 and Salmonella on almonds were reduced by plasma treatment as shown in Figure 6c, which reveals that plasma as a novel antimicrobial intervention for nuts is able to eliminate foodborne pathogens. Air plasma has a more effective inactivation on almonds than N_2, and both plasmas have minimal adverse effects on aroma, color, or surface features. Air plasma, as a CAP with oxygen-containing feeding gas, appears more effective and more importantly is attractive for economical large-scale industrial processing.[164,165] Figure 6d exhibits the effect of plasma on some bioactive compounds of acerola juice, where N_2 plasma has a positive influence on color, vitamin A, and carotenoids. The increase of vitamin A and carotenoids contents probably depend on the release of carotenoids and membrane bonded vitamins.[147] The effect of N_2 plasma on vitamin C was not significant, maintaining its retention above 95%. Separately, Sarangapani and Xu reported a decrease in vitamin C content of blueberry

fruits and orange juice, respectively, when treated with oxygen-containing gas.[166,167] This is because vitamin C is very sensitive to O_3 formed in plasma, but O_3 is not generated in N_2 plasma.[168] Moreover, plasma also works for fresh produce, killing a wide range of pathogenic microorganisms and stopping microbial proliferation in water with high nutrient content.[169]

3.2 Wound healing

The skin, a critical structure shielding internal tissues from microbial infection, mechanical damage, extreme temperature, and ultraviolet radiation, is the organ with the largest surface area of the human body. When the skin is injured, multiple cell types within epidermis, dermis and hypodermis require coordination of precise stages to achieve healing.[170] Changes in the microenvironment, such as oxygen levels, mechanical forces, extracellular matrix, chemokines, and growth factor synthesis, directly impact activation and cellular recruitment, potentially inducing wound healing at impaired states. Wound healing is an extremely complex process requiring several different cell types' intricate synchronization in sequential steps (inflammation, hemostasis, re-epithelialization, growth, and remodeling).[171] In addition, wound healing strongly involves the presence of reactive species in balanced content, where redox-sensitive and oxygen-dependent signaling represents an indispensable step in the healing. Since wound healing is related to redox control, there is a noticeable link to plasma medicine. Recently, CAP-generated reactive species were seen to act as mediators of a diverse set of cell responses that impact cellular structures, apoptosis, and differentiation. CAP supports wound healing processes by modulation of wound healing-relevant cell parameters: inflammation, proliferation, migration, and viability.[107] As a key action in the context of chronic wounds, plasma disinfection provides an effective treatment method. Isbary *et al*. conducted a clinical trial using CAP to treat chronic wounds. They found that CAP can kill a variety of pathogenic bacteria, reduce the bacterial load of wounds after treatment, and accelerate the healing of chronic wounds.[172] The possibility of a bacteriostatic mechanism of CAP has been reported, such as NO and NO_2^- and H_2O_2.[109]

It is relevant to the discussion of plasma control to mention tissue oxygen pressure. Oxygen partial pressure (pO2) is a key factor in cell regulation and plays a key role in cell and tissue physiology and pathology. Collet *et al.* found that pO2 remained high after plasma was applied to the skin.[173] The findings suggest that plasma-driven oxygen increases may pave the way for tumor blood

vessels to normalize and provide a valuable tool for combination therapy when used as adjunctive therapy. Kisch *et al*. found that oxygen saturation of tissues and postcapillary venous filling pressure increased after CAP treatment.[174] Repeated use of CAP can promote and prolong skin microcirculation to achieve the effect of promoting wound healing. Busco *et al*. studied the effect of oxygen dissolved in the media during CAP processing on the generation of reactive oxygen and nitrogen species (RONS).[175] They assessed the production of intracellular RONS after CAP treatment. The data show that plasma induced RONS in cells are affected by oxygen concentration, with higher oxygen concentration producing more RONS.

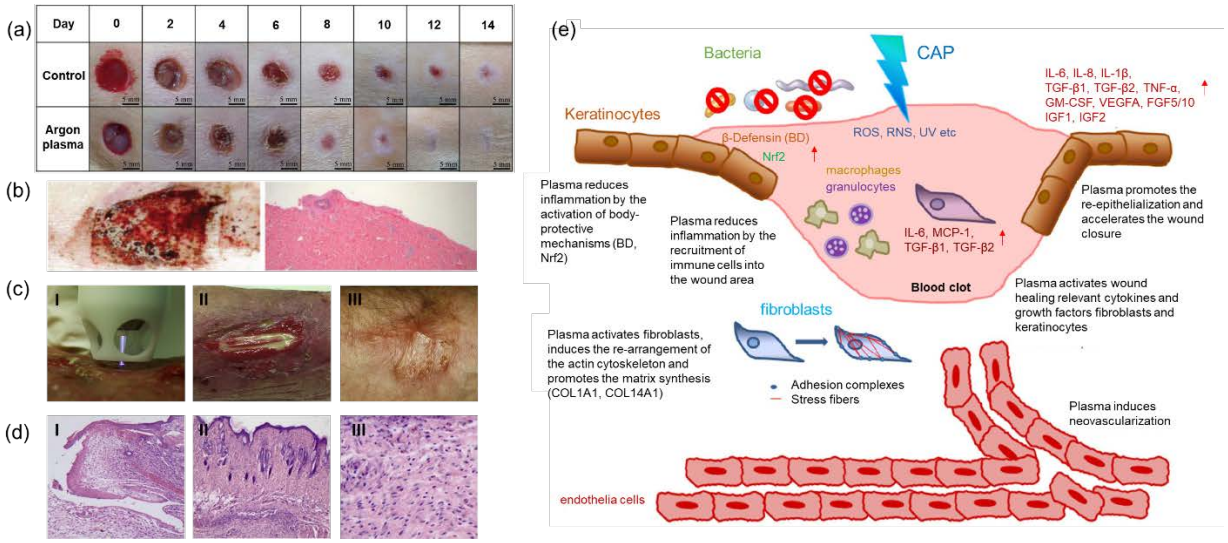

Figure 7. Plasma delivery for wound healing. (a) Effect of CAP treatment and healing time on kinetics of small wounds on streptozotocin-induced diabetic rats. Reproduced under the terms of the CC BY license.[176] Copyright 2018, Nature Publishing Group. (b) Representative and histological images of the wounded pig skin after plasma treatment: clot forming on the wound and no damage to normal tissue. Reproduced from reference.[177] Copy right 2011, Begell House Inc. (c) CAP treatment of wound healing disorders at radial forearm free flap donor sites on patient with partial split-thickness skin: plasma device (I), before treatment (II), and 6 weeks after treatment (III). Reproduced with permission.[178] Copyright 2017, Elsevier. (d) Histological microscopy images of wound tissue samples at day 7. Control wound (10× magnification) (I), wound treated for 30 seconds (10× magnification) (II), and wound treated for 30 seconds (40× magnification) (III). Reproduced with permission.[179] Copyright 2015, Wiley-VCH. (e) Wound healing potential mechanisms of the molecular and cellular effects mediated by plasma treatment. Reproduced with permission.[180] Copyright 2018, Elsevier.

Figure 7 shows plasma delivery for mice, pig, and human wound healing and the potential mechanisms of CAP for wound healing. Wound healing kinetics were examined for Type 2 diabetic mice treated by CAP as shown in Figure 7a, and plasma healing was much faster than that of the control groups. Wound contraction was highly distinguishable at day 6 after plasma treatment and was largely improved after day 9. The researchers also indicated that plasma treatment increased free radicals amounts to accelerate the healing process of diabetic and normal wounds through cell signaling and affecting some pathways. Plasma-generated species attack invading pathogens and bacteria, and excess ROS generate superoxide dismutase stimulating re-epithelialization and angiogenesis along with NO to promote healing.[181,182] Figure 7b displays wound surface and histological images of live pig skin exposed to plasma treatment. A short treatment of plasma on pig tissue resulted in blood coagulation and a thin layer of clear coagulum with no or minimal damage. This quickly-formed thin film of coagulated blood protected the wound from further external disturbances.[19,183] Plasma treatment does not induce any toxic effects to the tissue itself and is safe for wounded skin and living organisms.

Patients with wound healing disturbances exposed to CAP obtained complete wound closure after 6 weeks (as shown in Figure 7c) suggesting that plasma induces the growth and migration of cells in the complex process of wound healing and promotes neoangiogenesis.[184,185] This is a good example of CAP applied to human wound healing. From animal experiments to clinical applications (Figure 7a, 7b, and 7c), CAPs exhibit their potential applications on wound healing. Figure 7d shows histological microscopy images of wound tissue samples: control (I, 10× magnification), and samples after plasma treatment for 30 seconds at day 7 (II, 10× magnification; III, 40× magnification). Epidermal cell regeneration was still in process for control sample (I). Epidermal cells' widespread regeneration with mature differentiation, together with a lots of granulation tissue hyperplasia, fibroblasts, and compact collagen deposition can be observed in (II) and (III). The abundant RNS in CAP enhanced the NO content in the wounds and improved extracellular matrix regeneration to promote wound healing. It is also important to point out that appropriate doses of ROS delivery in CAP can promote the proliferation of repairing cells, facilitating wounds transiting into the remodeling period more quickly than normal and thus accelerating the wound healing, while excessive ROS doses will induce cell apoptosis or necrosis and have a negative effect on wound healing.[179]

Schmidt *et al*. indicates immunofluorescence staining of *Nrf2* distribution and nuclear expression in the dermal layer after plasma treatment and *Nqo1* majorly expressed in the epidermal layer.[186] The redox-regulating *Nrf2* signaling pathway is the pivotal involvement in plasma-treated wounds. *Nrf2* enables responses to oxidizing noxae as a gatekeeper of extra- and intra-cellular redox signals and controls antioxidant glutathione peroxidases and glutathione metabolism through glutathione reductase's transcriptional modulation.[187-189] During wound healing, plasma-induced protective *Nrf2* signaling counteracted oxidative stress through upregulating its down-stream targets including thioredoxins, peroxiredoxins, and catalase.[186] It also signals inflammation through inhibiting *NF-κB* and activating *HMOX1/COX2*, and *Nrf2-ARE*-driven genes such as *Nqo1* and *HMOX1* block monocyte chemoattractant protein-1.[190,191] *Nrf2* also plays a key role in regulating innate immune cells (macrophages) that respond to a repertoire of cytokines to enhance the inflammatory response.[192,193] Plasma upregulates pro-inflammatory markers *IL-6* or *TNF-α* and increases macrophages at the early stage of wound healing and enhances anti-inflammatory and antioxidant *Nrf2* pathways. Arndt *et al*. summarized that CAP mediated the molecular and cellular effects during wound healing, as shown in Figure 7e. Plasma accelerates wound healing through: (1) speeding up wound closure and promoting re-epithelialization, (2) decreasing inflammation *via* recruiting immune cells into the wound area and activating body-protective mechanisms, (3) triggering fibroblasts that promotes the matrix synthesis and induce the actin cytoskeleton re-arrangement, (4) activating cytokines/growth factors related to wound healing in keratinocytes and fibroblasts, and (5) inducing neovascularization.[180]

3.3 Blood coagulation

Coagulation is an important step of hemostasis and occurs immediately after damage to the vascular endothelium to prevent blood loss.[194] The natural tendency of blood to coagulate is of course one of the body's main mechanisms for combatting injury. However, with modern surgical and medical practice, attempts are often made to explicitly control blood coagulation rates: decreasing the tendency for the duration of surgeries, for specific health concerns, and related to medical implants; or increasing blood coagulation rates post-surgery for wound healing, in the event of major trauma, and for other purposes. While numerous tools exist currently for such purposes, including electric cauterization, various medications, thermal coagulation acceleration,

and others, most of these methods have various degrees of deleterious side effects that must be carefully managed. Plasma enters the field as a potential tool for controlling blood coagulation rates at specific locations with minimal side effects, as CAP triggers natural, rather than thermally induced, blood coagulation processes through selective actions of CAP on blood proteins.

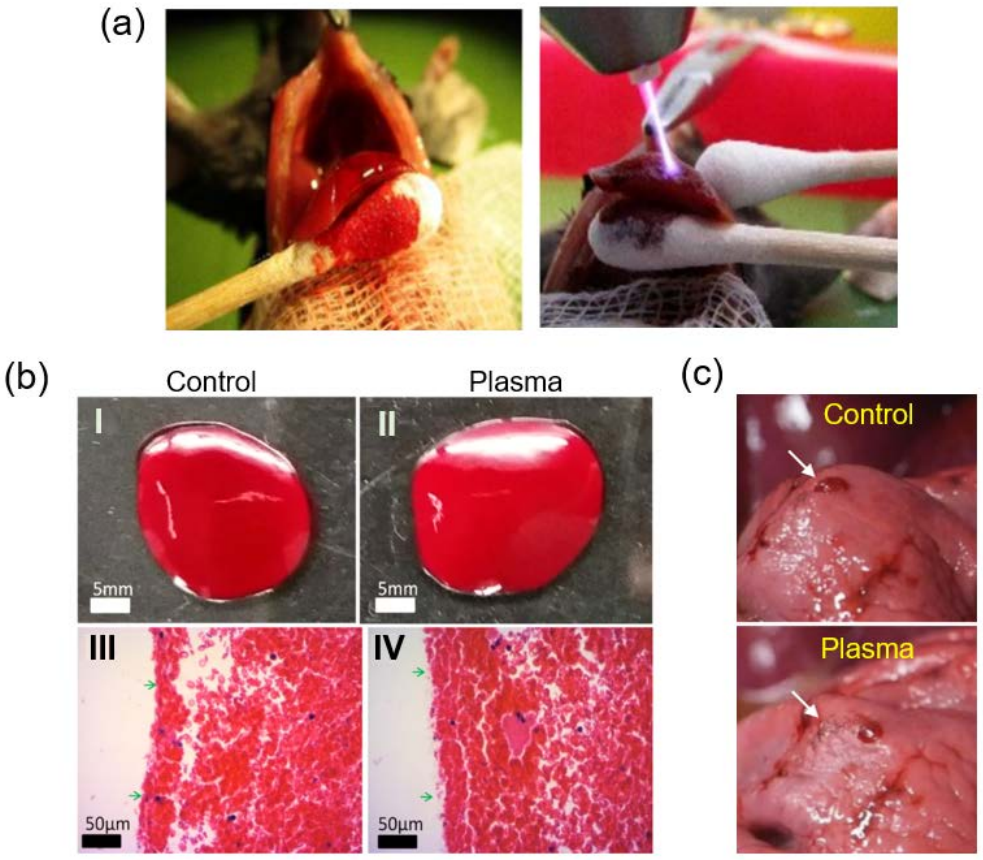

Figure 8. Plasma delivery for blood coagulation. (a) Plasma induces pro-coagulative responses in murine liver incision surgical procedures with a platelet-dependent manner. Reproduced with permission.[195] Copyright 2017, Elsevier. (b) Macroscopic appearance of blood coagulation without or with plasma treatment and the HE stained microstructure of the paraffin sections. Reproduced from reference.[194] Copyright 2018, IOP Publishing. (c) Plasma treatment for a bleeding lesion on porcine tissues. Gastric mucosa was observed before treatment and after plasma treatment. Reproduced with permission.[196] Copyright 2017, Elsevier.

The use of anticoagulants in patients is a common concern for surgery, as they can lead to an increase in blood loss during surgeries. One study comparing electrocauterization to CAP treatment both *ex vivo* and *in vivo* for mice also included subjects on two separate anticoagulants.[195] As shown in Figure 8a, CAP induces pro-coagulative responses in the murine liver incision

surgical procedures with a platelet-dependent manner. The argon-fed plasma jet drove coagulation that mimicked natural (control) coagulation in mice treated with plasmatic but not cellular inhibitors; plasma treatment likewise induced none of the tissue damage generally associated with electrocauterization.[195] In addition, Bekeschus *et al*. found plasma-mediated platelet activation inducing platelet aggregation.[197] They pointed out that the feed gas used determined the nature of platelet activation, finding out that wet and dry argon gases led to improved platelet activation and subsequently increased coagulation in comparison to oxygen- and nitrogen-containing gases. A separate study involving human subjects sought to determine the effect of nonthermal plasma treatment on coagulation times for individuals taking the anti-coagulant Warfarin.[198] While median untreated clotting times were 88.5 and 48 minutes between the Warfarin and control groups respectively, plasma treatment resulted in median clotting times of 22.2 and 19.2 minutes, indicating that the presence of the anticoagulant had minimal effect on the ability of CAP to drive clotting, possibly due to hemolysis-based clotting mechanisms described previously.

CAP appears to induce fibrinogen cross-linking in exposed samples, with the results at least partly mediated by H_2O_2 production from the plasma discharge as well as the destruction of red blood cells to release heme into the target area.[199] A more thorough analysis revealed that current and specific plasma device design could be controlled to induce either classic whole-blood clotting without hemolysis or to increase red-blood-cell driven clotting *via* hemolysis as desired;[200] in a separate study, the same investigators found that the supplied power to the CAP device was critical in initiating clotting, with the specific levels (6.7-7.4 kW) likely dependent on the tissues and other conditions being treated.[201] Yan *et al*. employed a homemade pulsed CAP jet for blood coagulation and applied it to rat hepatectomy.[194] Plasma can lead to rapid blood coagulation. Compared with the control sample, the plasma-induced blood coagulation layer is thicker and denser (as shown in Figure 8b). Rad *et al*. determined that a treatment duration of 8.6 seconds by He plasma was sufficient for complete coagulation in a mouse liver model.[202] Nomura *et al*. investigated plasma treatment for a bleeding lesion on porcine tissues. It was observed that plasma treatment could quickly control the bleeding lesions of porcine liver and stomach, and the tissue damage caused by plasma treatment was not detected under the optical microscope (as shown in Figure 8c).[196]

A general comparison of the application of CAP (17 kV, distances of 2 cm and 4 cm) *via* a plasma needle to extracted blood samples from 70 random patients resulted in decreased mean blood clotting time (1.5 min and 2 mins, respectively, compared to 3.2 minutes prior to treatment) and reduced prothrombin time (14 and 14.8 seconds, respectively, compared to 15.4 seconds prior to treatment), with an analysis of blood proteins indicating an acceleration of natural, rather than thermal, blood coagulation.[203] A study using a CO_2-based plasma jet for inducing hemostasis during endoscopic surgery on pigs resulted in successful coagulation and stasis reacted after 70 ± 20 s with no thermal or other tissue damage.[204] A detailed analysis of the results of plasma treatment using various feed gases may elucidate some of the specific effects that plasmas have on red blood cells and platelets. Kim et. al performed such an analysis using DBD with air, N_2, and Ar feed gases and various preparations of human blood samples.[205] They found that platelet function, blood coagulation, and hemorheological properties were affected in different ways depending on the specific conditions of the test, sometimes in contrast with prior studies. In general, air plasma had the most variability from controls in results, both beneficial and less desirable, with a dose-dependent effect that could be controlled based on time of exposure; the presence of oxygen in the air as part of the feed gas is likely responsible, as various ROS were more readily and easily formed in the plasma. The authors also suggest that the nature of the DBD plasma device, which provides for a substantially larger contact area and thus higher infusion of radicals into the sample, may be partly responsible for the variation from other studies that mostly use various forms of plasma jets.[205]

Related to surgeries, coagulation, and clotting around artificial implants including lines introduced into veins and arteries for long-term use as delivery pathways (such as PICC lines for chemotherapy treatments) are important considerations related to blood coagulation. In this case, it has been proposed that, among various factors, the surface hydrophilicity of the implant is one of the most critical characteristics that can affect clotting at the point of contact between the implant and the bloodstream. Bekeschus *et al*. investigated the molecular and physicochemical mechanisms of platelet activation induced by gas plasma treatment.[206] Untreated or argon gas-treated blood incubated with lysed blood showed a strong platelet-activation differing to an unimportant, minor extent to that of gas plasma-treated blood. Plasma releases ROS, but neither the addition of chemically-generated ROS nor the removal of long-lived ROS can recapitulate or

abrogate the gas plasma effect, respectively. However, compared with whole blood treated with gas plasma, platelet activation in platelet-rich plasma was significantly impaired, and the whole blood contained a large amount of hemoglobin, indicating the presence of erythrocytes (hemolysis). All blood was incubated with a concentration-matched hemolytic agent to replicate gas plasma mediated platelet activation. Junkar elaborates at length on the various mechanisms by which clotting and cellular adhesion appear to be affected by plasma treatment, but an increase in hydrophilicity to the point of unmeasurable water contact angle and near-zero platelet adhesion after 30 s of treatment is perhaps the most significant result with regards to blood clotting.[207] The mechanism for water contact angle was similar to that in other applications, namely an increase in O presence and decrease in C presence on the surface of the implant; this implies improved reduction in clotting for implants treated with plasmas using O_2 or O-laden feed gases.

As stated, CAP has been shown to drive more natural coagulation and clotting in subjects even in the presence of many anticoagulants, with reduced clotting times but without the tissue damage associated with more traditional methods like electrocauterization. It can also be used to reduce coagulation in specific settings as desired, with various characteristics of the plasma (including power, feed gas, and even device type) capable of being tuned to meet specific requirements for treatment.

3.4 Oral and dental treatment

CAP has been applied for oral and dental treatment due to its antimicrobial properties and its targeted cell death mechanisms.[208] Studies of CAP have shown expected results in tooth biofilm inactivation, tooth bleaching, prevention and treatment of oral diseases, and freshening breath. The applications of plasma in dentistry vary considerably. The primary uses are biological (sterilization, tissue improvement, etc.) and surface or materials modifications (such as wettability/adhesion improvement, osseointegration, whitening, and polymerization). The majority of applications rely on the generation of ROS for both bactericidal effects and improved hydrophilicity, while the generation of H_2O_2 *via* plasma discharge in a humid environment can be leveraged for bleaching and other purposes. The general mechanisms by which CAP can inactivate bacteria and other organisms have been covered elsewhere in this review.

For oral health, particular importance is placed on CAP's effects on oral biofilms that persist in human oral cavities. While normally in homeostasis, biofilms can be perturbed by bacterial infections or other changes in oral conditions, leading to multiple negative consequences including dental plaque. Two-minute exposure to an argon-oxygen CAP has been shown to substantially reduce the presence of bacteria in oral biofilms.[209] A study by Zhang *et al*. found that plasma treatment after traditional scaling and root planning led to decreased bone loss and inflammation in a rat model of periodontitis versus the untreated group.[210] Even indirect exposure to plasma-generated reactive species can reduce the presence of oral pathogens, with a 60-second rinse of plasma-activated water in place of mouth wash showing significant reductions in several common oral pathogens *in vitro*, largely through the actions of atomic oxygen and hydroxyl radicals (Figure 9a).[211]

The ability of CAP to increase the wettability of a surface and, consequently, increase surface adhesion has been demonstrated. In one study, 30 seconds of argon-fed plasma treatment improved bonding between dentin and common dental adhesives, with measurable increased micro tensile bond strength identified after two years of storage in water.[212] An *ex vivo* study by Yeter *et al*. found that 30 seconds of argon-fed plasma exposure improved push-out bond strength for a ceramic-based sealer but not an epoxy-based sealer.[213] Dentin tubule penetration was shown to be improved for certain root canal sealers when the tooth was treated with argon-based plasma prior to filling.[214] A study has shown that zirconia, a commonly used material for tooth restoration, had significantly reduced contact angle and significantly greater shear bond strength after plasma treatment.[215,216] Also as a potential replacement for metal implant material, zirconia was limitedly researched under plasma treatment. Zheng *et al*. found that DBD plasma treatment of zirconia enhances its biocompatibility with human gingival fibroblasts.[217] Shon *et al*. found that the He plasma treatments on powder-injection molded (PIM) zirconia implants made a more hydrophilic surface and enhanced the bone integration of the implants without changing the micro-topography.[218]

As mentioned, osseointegration for dental implants can also be improved by plasma treatment. CAP has long been known to affect surface roughness and other characteristics for various materials, and its effects on titanium and titanium alloys used for dental implants are similar. For

example, treatment with a nitrogen-fed plasma jet for 10s led to not only a significant reduction in surface bacterial adhesion for Ti and Ti-Ag treated implants but also increased cell attachment without changing the roughness or topology of the implants (Figure 9b).[219] The changes in adhesions for both bacteria and tissue were determined to be a result of hydrophilicity changes, as the bacteria tested was specifically hydrophobic while cellular tissues are hydrophilic. This presents a challenge, as hydrophilic bacteria would also likely experience increased attachment due to such a treatment. The wettability changes induced by CAP on titanium and zirconia dental implants have been shown to be similar to those induced by 12-minute ultraviolet light exposure with minimal degradation or changes to surface topology.[220]

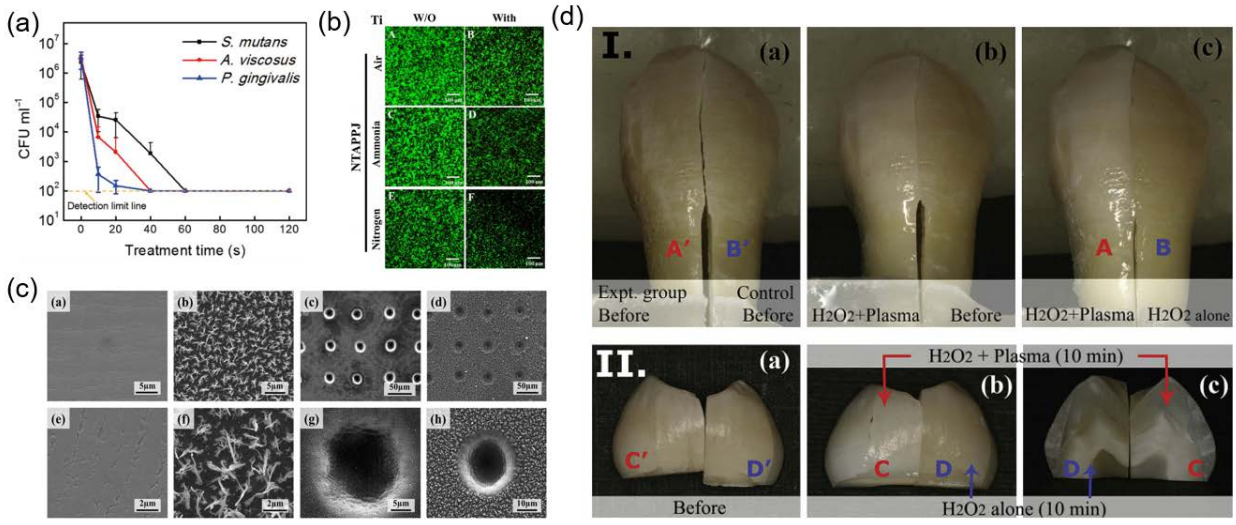

Figure 9. Plasma delivery for oral and dental applications. (a) Disinfection efficacy evaluation of the plasma-activated water (PAW) treatment group. A. viscosus, Actinomyces viscosus; CFU, colony-forming units; P. gingivalis, Porphy romonas gingivalis; S. mutans, Streptococcus mutans. Reproduced with permission.[211] Copyright 2017, Wiley-VCH. (b) Confocal laser microscopy images following live (green) and dead (red) staining of Streptococcus sanguinis adherent to Ti specimens without (w/o) (A, C, E) or with (B, D, F) non-thermal atmospheric pressure plasma jet (NTAPPJ) pretreatment using the three working gases (air, ammonia or nitrogen). Representative images of independent triplicate experiments are shown. Reproduced with permission.[219] Copyright 2017, Elsevier. (c) SEM images of non-treated, plasma-treated, laser-treated, and plasma and laser-treated poly-ether-etherketone (PEEK). Reproduced with permission.[221] Copyright 2013, Elsevier. (d) The external bleaching effect of plasma treatment (I). The internal bleaching effect of plasma treatment (II). Reproduced with permission.[222] Copyright 2009, Elsevier.

Likewise, poly-ether-etherketone (PEEK) as another potential replacement for metal implant structures has also seen limited research under plasma treatment (Figure 9c). Mahrous *et al*. compared integration of treated and untreated PEEK surfaces into canine femurs after 12 weeks of growth and determined a significant increase in bone-to-implant contact for plasma-sprayed PEEK surfaces.[223] A separate study compared the effects of ultraviolet (UV) light and oxygen-fed plasma on Ti, Zr, and PEEK surfaces, with oxygen plasma and UV showing preferential cell adhesion over argon plasmas and untreated samples; different cell types expressed differences in preference to UV or O-plasma treatment.[224] Singh *et al*. presented a review of plasma treatment of PEEK in a broader context including dental implants.[225]

While largely cosmetically, stain reduction and tooth whitening are common needs where CAP can also be employed in dentistry (Figure 9d). The safety and efficacy of tooth bleaching or whitening *via* CAP have been demonstrated multiple times.[226,227] For example, one *ex vivo* study on sample human teeth found that a 20-minute application of helium-fed plasma in combination with periodic saline-solution application to maintain moist conditions had superior bleaching results with lower enamel damage than more traditional H_2O_2 gel;[228] enamel etching present in the plasma-treated sample was posited as resulting from peroxide created through the reaction of the plasma with the saline solution. A sample case study from Croatia showed the application of CAP without bleaching agents to significantly improve the appearance of a tooth *in vivo* over the course of 4 60-second treatments at one-week intervals.[229]

As a preventative measure, the use of a "plasma brush" to apply a stain-resistant SiO_2-like coating to samples of bovine teeth and resin composites lead to a reduction in color changes due to various common liquids as well as an increase in measured hardness.[230] Separately, CAP treatment in combination with 15% carbamide peroxide resulted in reduced susceptibility to red wine staining after treatment when compared with H_2O_2 bleaching.[231] These results show that, in addition to effective tooth whitening with lower enamel damage, the benefits of plasma treatment can extend well after the treatment itself to reduce the frequency of future treatments.

CAP has been shown to have significant advantages over traditional methods in oral health applications. While some aspects of treatment, such as the feed gas used and the duration of

treatment, need to be considered in light of the specific desired results, the reactive species generated within the plasma discharge can reproduce the effects of multiple different traditional compounds ranging from sterilization, whitening, and even strengthening of adhesion and surfaces. Plasma treatment promises to be a powerful tool to be commonly used in dentistry in the near future.

3.5 Cancer therapy

Cancer is a large group of diseases involving abnormal cells growing uncontrollably to invade adjoining parts and/or spread to other parts of the body. Current cancer treatment methods mainly include surgical resection, radiotherapy, chemotherapy, and photodynamic therapy. However, next-generation treatments with enhanced efficacy and limited side effects are continuously being explored.[232-235]

Recently, the potential of CAP applications for cancer therapy have been developed. Plasma treatment can induce selective cancer cell death *in vitro* and tumor size reduction *in vivo*.[16,236,237] Plasma technology can also achieve non-invasive or minimally invasive treatment, accurate positioning, drive a radical or cytoreductive effect on tumors, and enhance the anti-tumor immunity of living organs.[238] The efficiency of CAP for cancer therapy is attributed to its ROS, RNS, and electrons, primarily related to apoptosis induction in tumor cells.[239,240] CAP potentially offers a minimally invasive surgery option, allowing specific cells to be removed without influencing the surrounding tissue. In the 2010s, Vandamme *et al.* performed pioneering work in *in vivo* antitumor action via plasma treatment of U87-Luc glioma tumors using pulsed DBD plasma.[241] The results showed that the mice well-tolerated plasma treatment and the tumor volume decreased after plasma treatment. Keidar *et al.* treated cancer cells *in vivo* and *in vitro* by CAP jet, and explored that CAP could selectively kill cancer cells without harming adjacent normal cells.[242] Recently, Metelmann *et al.* published a report on the anticancer effect of CAP in different animal models and cell lines, demonstrating that CAP can not only change the surface of tumor,[243] but also provide lasting partial remission in anticancer therapy. From the website of US Medical Innovations, LLC (USMI), they announced results of the first phase clinical trial approved by the FDA, which evaluated the therapeutic effect of CAP on stage IV relapsed metastatic solid tumors

(FDA IDE #190165) and their recently discovered mechanism of CAP-induced apoptosis in breast cancer.

In the area of neurodegenerative diseases, CAP has shown potential regarding forced differentiation of neural cells. The results of the study by Tanaka *et al*. showed that plasma-activated medium could induce more intracellular ROS in glioblastoma multiforme (GBM) cells and elevate stress-inducible genes such as GADD45α signaling molecule to induce cell apoptosis.[244] Another study found after U37MG GBM cells were exposed to 75 KV CAP for 3 minutes, 50% cell death was observed after 5 hours, and the viability was less than 20% after 24 hours.[245]

Chen *et al*. reported the use of the portable air-fed CAP (aCAP)device for CAP-mediated postsurgical cancer treatment (Figure 10).[246] Inspired by lightning (a giant spark of electricity with gigajoule energy in the ambient air), they devised a portable and lightning-mimicking CAP (LM-CAP). The working mechanism and design rationale of the LM-CAP device are illustrated in Figure 10a. LM-CAP device applied on residual tumor cells at the surgical cavities, ROS/RNS induces cancer immunogenic cell death (ICD) and releases tumor-associated antigens (TAAs) in situ, evoking effective antitumor immunity. Immature dendritic cells (DCs) will engulf and process TAAs into peptides when migrating to the tumor-draining lymph nodes, where mature DCs present antigenic peptides to T cells and produce cytotoxic T cells. Then, the subsequent T cell–mediated immune response can inhibit the growth and recurrence of tumors (Figure 10b). They evaluated the performance of LM-CAP treatment *in vivo* using an incomplete-tumor-resection model to mimic the postsurgical local relapse. Figure 10c shows Increased levels of calreticulin (CRT) in the residual tumor cells in the mice receiving LM-CAP treatment, demonstrating that LM-CAP treatment could induce ICD *in vivo* as well. Mice treated with LM-CAP showed remarkly improved tumor regeneration control compared with the surgery-only group. Correspondingly, survival was significantly prolonged in mice receiving LM-CAP treatment, and longer treatment duration led to better outcomes (Figure 10d). Over forty percent of mice survived for at least 60 days when treated with 4 min of LM-CAP. The body weights of mice were not impacted during treatment (Figure 10e). In this proof-of-concept study, the longest post-surgical LM-CAP treatment was set at 4 min. We could expect that the prolonging the treatment time or repeated

treatment of LM-CAP can further improve the therapeutic effect. After incomplete tumor resection, aCAP treatment helps to inhibit the prolonging survival and growth of the tumor in B16F10 melanoma and 4T1 breast tumor models.

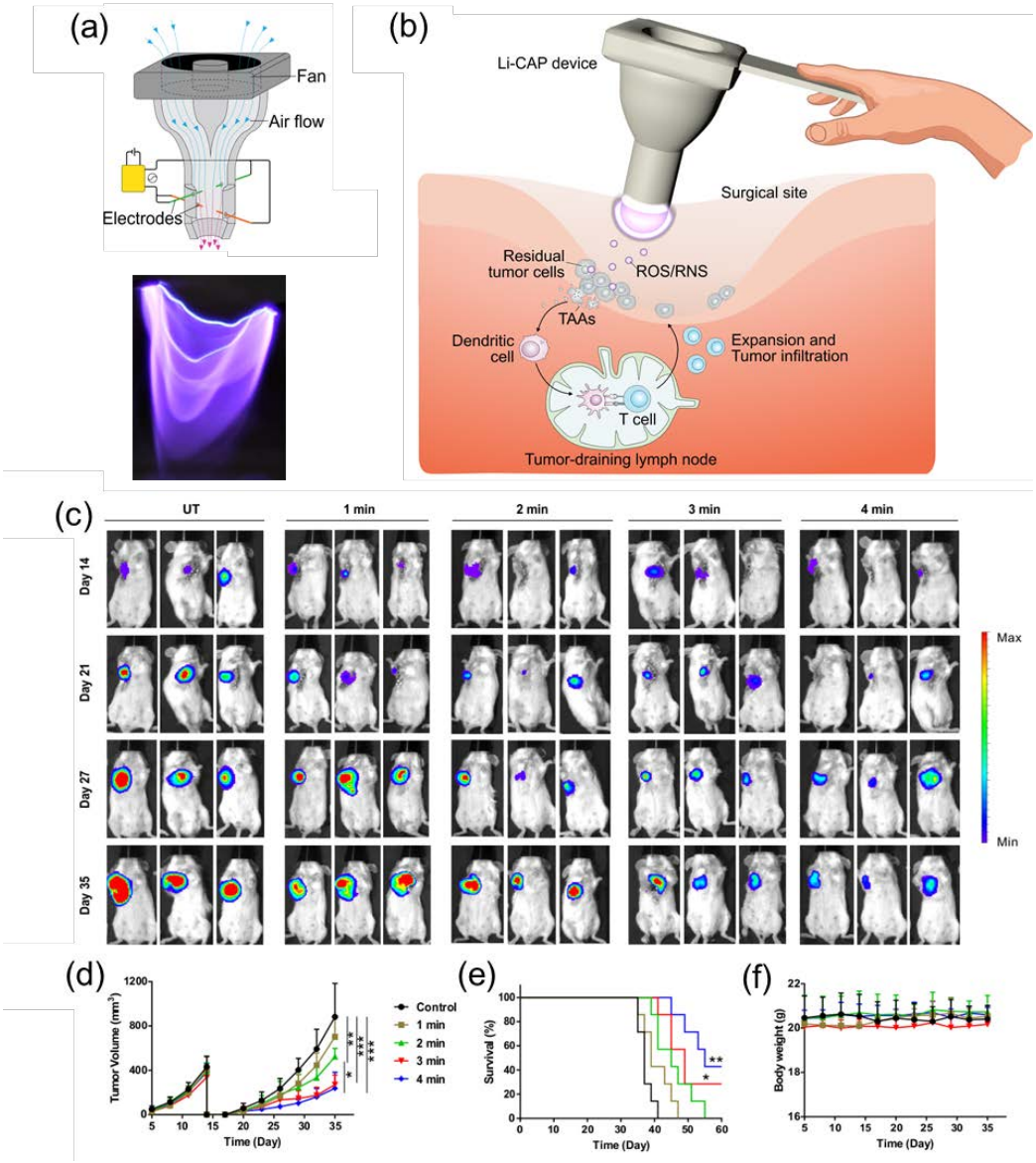

Figure 10. Portable air-fed CAP device for postsurgical cancer treatment. (a) Schematic of the working mechanism for the LM-CAP device and a photograph of plasma discharge containing lightning (spark) at joule energy level. (b) Portable LM-CAP applied in the surgical cavity and resulted in cancer immunogenic cell death (ICD) of the residual tumor cells. Tumor-associated antigens (TAAs) from dying cells presented by DCs to T cells in the tumor-draining lymph node, inducing the generation of tumor-specific cytotoxic T lymphocytes (CTLs) to combat tumor cells. (c) The bioluminescence image of 4T1 tumors after removing the primary tumor. Tumor resection was done at Day 14. (d) Average growth kinetics of tumors in

experimental groups (n=7). Growth curves stopped once the first mouse died. (e) Kaplan-Meier survival curves for control and treated and mice (n=7). (f) Mice body weight changes in each group after treatments. Reproduced under the terms of the CC BY license.[246] Copyright 2021. AAAS.

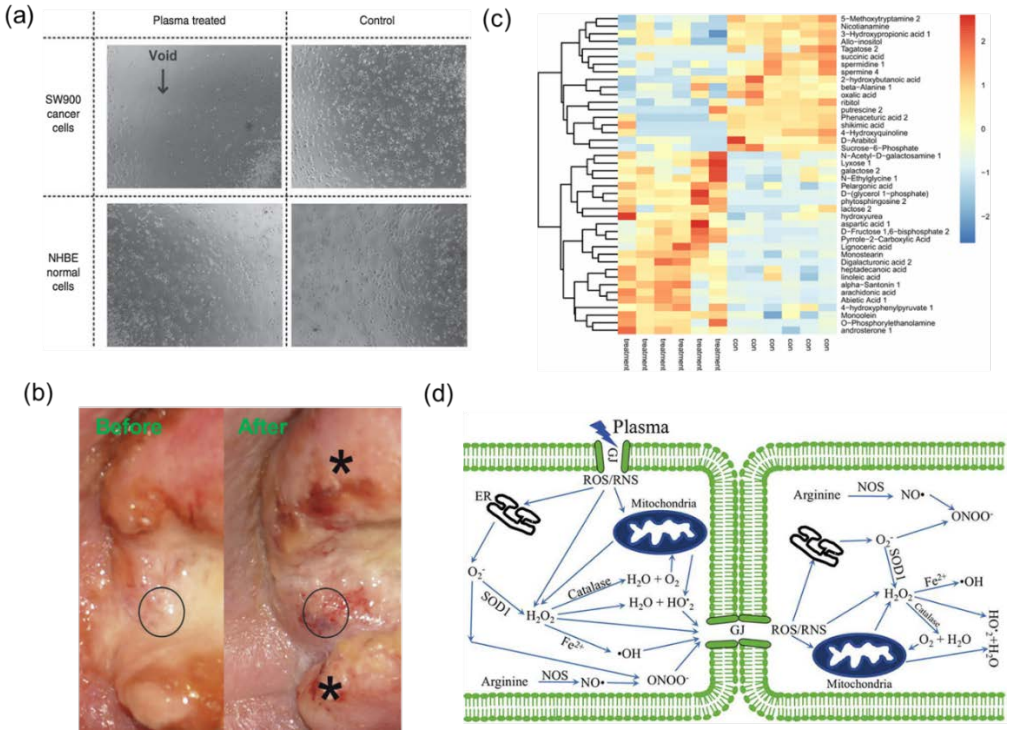

Figure 11. Plasma delivery for cancer therapy. (a) Plasma selectivity effect: cancer cells (SW900) detached from the plate after plasma treatment, while no detachment of the normal NHBE cells after plasma treatment. Reproduced under the terms of the CC BY license.[242] Copyright 2011, Nature Publishing Group. (b) Plasma was applied for clinical head and neck cancer, and the tumor surface response appeared a flat area with vascular stimulation (a flat surface aspect with stimulated vessels within the circle in comparison with areas of tumor progress (*) in the untreated surrounding areas). Reproduced with permission.[247] Copyright 2016, Elsevier. (c) Heatmap of hierarchical clustering analysis of human myeloma cells treated by CAP and control group. Reproduced under the terms of the CC BY license.[248] Copyright 2018, Springer. (d) Plasma-generated ROS induced bystander effect *via* gap junction intercellular communication. Reproduced under the terms of the CC BY license.[249] Copyright 2019, Taylor & Francis Group.

Keidar *et al*. investigated the effect of CAP on SW900 cancer cells and normal NHBE cells and pointed out a strong selective effect as shown in Figure 11a. After plasma treatment, 60-70% of SW900 cancer cells were detached from the plate while no normal NHBE cells were detached. Plasma treatment caused a significant reduction in SW900 cancer cell count, while normal NHBE

cell count remained unchanged. Plasma selective killing has been confirmed by many scientists.[250-252] Schuster *et al*. applied plasma to 12 patients with head and neck cancer as shown in Figure 11b. After plasma treatment, apoptotic cells were detectable, and the tumor surface appears as a contraction of tumor ulceration rims or a flat area with vascular stimulation. CAP applied to patients is a big milestone for plasma clinical applications.

Scientists are actively working to identify the mechanisms of CAP for cancer therapy. Xu *et al*. employed bioinformatic analysis to determine the metabolism pathway affected by plasma treatment (as shown in Figure 11c). The results of hierarchical clustering analysis were visualized in a heatmap to show obvious differences in metabolic grouping patterns of plasma-treated offspring. Cellular metabolism systematic reprogramming helps tumor cells' aberrant proliferation, involving the generation of energy required for the intracellular redox status regulation, cell division, breakdown nutrients, and synthesis nutrients.[253] Redistributing metabolites flux maintain the cells' malignant transformation phenotype through corresponding metabolic pathways.[254] The most significant change of myeloma cells after plasma treatment is β-alanine metabolism pathway.[248] Xu *et al*. reported reactive species generated in CAP triggered the bystander effect based on the intercellular communication of gap junction through employing reactive molecular dynamics simulations (Figure 11d). HO_2^* and •OH radicals were able to chemically react with the gap junction's N-terminal to induce structural damage. They identified two breaking mechanisms: HO_2^* radicals damage C-C bonds and •OH radicals damage C-N peptide bonds. Now, molecular dynamics simulations are important tools employed to explain the mechanisms of plasma cancer treatment. Some of the results indicate that plasma-generated species interacting with cell membrane through oxidation of the phospholipids induce an overall increase in the bilayer disintegration, pore creation, and membrane permeability.[255,256] Molecular dynamics simulations also explain that reactive species destroy DNA/RNA, linolenic acid, and proteins through damaging bonds.[257-259]

Plasma-generated reactive species include nitrite (NO), superoxide (O_2^-), ozone (O_3), atomic oxygen (O), singlet delta oxygen (SOD, $O_2(^1\Delta g)$), •OH, and others; plasma-induced intracellular reactive species normally include protonated perhydroxyl radical (HO_2^*), O_2^-, nitric oxide ($^*NO$), •OH, H_2O_2, peroxynitrite ($ONOO^-$), nitrite (NO_2^-), etc.[260] O_3 is known to have a strongly aggressive

effect on cells, and O is believed to have a significant effect on cells. O_2^- can activate mitochondrial-mediated apoptosis through changing the potential of the mitochondrial membrane and simultaneously regulating pro-/anti-apoptotic genes for caspases activation.[261] Additionally, O_2^- is generated in the mitochondria through O_2 capturing electrons from the electron transport chain, which is able to rapidly convert to H_2O_2 by superoxide dismutase (SOD). $O_2(^1\Delta g)$, another important plasma-generated species, not only generating oxidative damage but also selectively killing tumor cells.[262] For oxidative proteins pathways, electrons transfer from PDI (protein disulfide isomerase) to O_2 and ERO-1 (endoplasmic reticulum oxidoreductin-1) through a FDA (flavin adenine dinucleotide)-dependent reaction.[263] Electron transfer suggests that ERO-1 strongly relates to protein load in the ER (endoplasmic reticulum) and induce ROS generation. •OH derived amino acid peroxides are able to induce cancer cell injury.[264] In addition, H_2O_2 can be converted into •OH owing to the presence of transition metals (such as Cu^{2+} or Fe^{2+}) and then damage gap junctions, lipids, DNA, and proteins due to its highly reactive nature.[249] $^*NO$ is produced from arginine by NOS (nitric oxide synthase), and reacts with O_2^- to form $ONOO^-$.[265] $ONOO^-$ is able to modify the function and structure of proteins and is highly destructive to tumor cells. Plasma-generated reactive species play major roles in what is referred to as redox or oxidation-reduction biology in plasma medicine. Convincing evidence of CAP selectivity towards tumor cells opens an important door for cancer therapy and bring hopes to people suffering from cancers.

3.6 Other applications

In addition to biomedical applications as discussed above, CAP works for immunotherapy, cosmetics, dermatology, gynecology, tissue regeneration, pulmonology, traumatology/orthopedics, maxillofacial surgery, ophthalmology, otorhinolaryngology, gastroenterology, and purulent peritonitis treatment. An in-depth discussion of each would be prohibitively ambitious, so instead, some of the more recent advances and research for a wide range of uses are included below. The search for noninvasive but effective methods for reducing or eliminating cosmetic blemishes and rejuvenating skin and subdermal tissues constitute a significant worldwide industry, and nonthermal plasma has several potential uses within this subdomain. As one example, the ability of CAP to induce skin regeneration has been linked to increased cellular regeneration and transcription by translocation of *β-catenin* into nuclei (Figure 12a).[266] This translocation has

previously been demonstrated to be one of the multiple pathways responsible for the proliferation of hematopoietic stem cells without increasing mutation or differentiation.[267] One unexpected side-effect of nitrogen plasma dermal treatment for rejuvenation was the apparent increase in hair follicle diameter for treated samples in a Wistar rat model, in addition to the measured dermal thickening and collagen formation;[268] while the effects on follicles seemed to be temporary for the study in question, any ability to restore or improve hair growth on treated skin could have wide-ranging cosmetic applications.

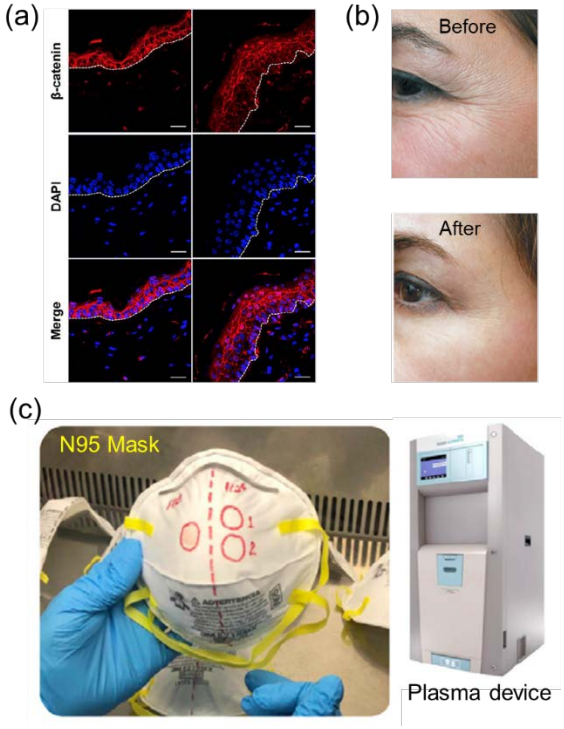

Figure 12. Plasma delivery for other applications. (a) Skin renewal activity of nonthermal plasma through the activation of β-catenin in keratinocytes. Reproduced under the terms of the CC BY license.[266] Copyright 2017, Nature Publishing Group. (b) Plasma for skin regeneration and cosmetology: before and after photos showing improvement on periorbital wrinkles (1 year). Reproduced with permission.[269] Copyright 2008, Wiley-VCH. (c) Disinfection of N95 masks artificially contaminated with SARS-CoV-2 and ESKAPE bacteria using hydrogen peroxide plasma. Reproduced with permission.[270] Copyright 2020, Elsevier.

In dermatology, CAP is also being investigated for specific applications such as pain or irritation treatment, scar reduction or removal, and the mitigation of skin aberrations such as actinic keratosis and epidermal barrier defects. For example, a recently-concluded randomized study using argon-fed plasma twice weekly for a 3-minute dose versus 3% diclofenac gel twice daily over

three months towards the reduction of actinic keratoses resulted in similar lesion reduction but significantly increased skin area affected by plasma treatment with a likewise similar reduction in adverse effects.[271] With skin wounds, plasma treatment was observed to reduce type I collagen and alpha-SMA levels during healing, leading to decreased scar formation without inducing other damage.[272] Epidermal barrier defects are often the result of pH imbalance in the skin, with higher pH and reduced acidity leading to bacterial infiltration; the reactive species generated by CAPs have been shown to increase skin acidity and decrease pH, leading to the destruction of invading bacteria and restoration of the epidermal barrier.[273] In another application, skin permeability was increased through employment of various DBD operational configurations, allowing for increased transdermal delivery of NaFluo as a model substance; the authors noted that treatment parameters may be modifiable to either increase or decrease permeability as needed for various medical situations.[274] Figure 12b shows expected improvements in periorbital wrinkles after plasma treatment. Histological studies performed on plasma-resurfacing patients have confirmed after one year of treatment, collagen continued to be generated, elasticity decreased and skin gradually returned to the youth. The US Food and Drug Administration has granted 510 (k) plasma skin regeneration clearance for treatment of wrinkles of the body, actinic keratoses, seborrheic keratoses, superficial skin lesions, and viral papillomata. Plasma offers a unique form of non-ablative resurfacing and is not chromophore dependent without inducing vaporization of the epidermis, leaving a layer of intact desiccated epidermis acting as a natural biologic dressing and promoting rapid healing.[269]

Biocompatibility, wear, and the integration of implants into existing bone structures are major areas of concern in orthopedics. As mentioned regarding dentistry, using CAP to both develop and treat implants can be of great benefit. For example, a hand-held plasma device was used to modify the surfaces of both metallic and non-metallic materials used in artificial joints and discs, with results that showed a 98% reduction in wear and 200-300% improvement in biocompatibility when compared to non-treated samples.[275] Similarly, nanographene oxide-reinforced hydrogel modified by cold microplasma to increase crosslinking demonstrated cytocompatibility and increased hyaline cartilage formation both *in vitro* and *in vivo*.[276] Biodegradable polymeric materials have also been treated as scaffolding to be used for the directed regrowth of bone tissue.[277]

Other more generalized effects related to interactions of reactive species with general cell mechanisms are also being studied and applied. Among other beneficial effects, plasma treatment has the potential to help reduce the negative consequences of oxygen and glucose deprivation during ischemic strokes.[278] This was theorized to be the result of increased NO presence within the cell after treatment, providing a reduction in mitochondrial apoptosis. The same team had found similar protection against oxygen and glucose deprivation for cardiac myoblasts, implying a generalized effect of low-level NO increases on cell health by preventing such deprivation under stress conditions.[279] Additionally, with the growing body of evidence that ROS and RNS play important roles in stem cell differentiation, CAP also has significant potential with regard to tissue engineering.[280]

With the COVID-19 pandemic still being a worldwide concern, the specific uses of CAP in relation to SARS-CoV-2 and similar pathogens are worth exploring. While the suddenness of the emergence of COVID-19 means that much of the research on the virus and subsequent disease is still being conducted, a large body of preprint and pre-reviewed work has been presented in the interest of international health and collaboration. As one example, a comprehensive review and prospective analysis on plasma's role in mitigation and treatment has been released, including speculation on future directions.[281] Much of the potential for COVID-19 disinfection or inactivation *via* plasma remains speculative or based on models using other viruses, especially coronaviruses. For example, while waterborne transmission of COVID-19 is not currently a primary concern, ozonation *via* DBD of water samples contaminated with coronaviruses has been proved to be effective as a disinfection method that may be applicable for the current crisis.[282] Other avenues of CAP use could also prove beneficial, such as the use of plasma-activated water in vaccine preparation.[283]

The disinfection of supplies, especially personal protective equipment (PPE) such as masks, is a primary concern in medical facilities to not only reduce cross-contamination but also conserve supplies when supply chains may be disrupted. The ability of a non-atmospheric hydrogen peroxide plasma to inactivate SARS-CoV-2 artificially placed on N95 masks has been demonstrated in research;[270] this portends well for CAPs, which are known to produce hydrogen peroxide when used with humid feed gases. The rapid increase in the use of 3D-printed supplies,

including masks, petri dishes, and others, has led to an increased need for contamination prevention as well as sterilization. A research team from Spain demonstrated the ability of an N_2-fed APPJ to modify the surface of petri dishes printed in polylactic acid and reduce the adhesion of biofilms, which could prove an additional protective measure against pathogens such as COVID-19 (Figure 12c).[284]

In short, research into these and other implementations of CAP is currently a major endeavor involving scientists and engineers around the world and in many different fields. Studies range from the basic science of generating CAPs and the effects of specific devices or conditions on resulting species to extremely targeted applications within narrow fields. This breadth of research is only likely to grow as understanding of plasma's interactions with and effects on biological and other systems increases over time. It is an exciting time for the science of plasma medicine.

4. Delivery of plasma-activated media

Recent studies reported that CAP affected cells or tissues not only directly, but also through the indirect treatment of cells or tissues with previously plasma-activated media (water, saline solution, cultured medium, etc.).[285-287] Indirect treatment could make up for the shortcomings of direct treatment, such as limitation to surface skin and plasma operating space. The effectiveness of plasma-activated media also improves the potential of food health and clinical biomedical applications because such media contains ROS and RNS that can be produced in advance and stored until use.[107,288]

4.1 Plasma-activated water

Plasma-activated water (PAW) represents an environmentally friendly and cost-effective solution.[289] Plasma treatment of water results in changes in the redox potential, conductivity, and the formation of ROS and RNS. The chemical composition of PAW is different from that of water, and PAW can be used as an alternative method for biomedical applications. Figure 13 represents the process of PAW generation and its biomedical applications. Optical photographs of various plasma discharge modes generating at the interface of water corresponding to the specific voltage/current conditions were revealed in Figure 13a, and their complexes consist of confocal and radial lines of different densities. The discharge modes can be divided into four specific stages: stage I (single filament), stage II (heat radiation), stage III (transition), and stage IV (multi-filament). The discharge stretches out to a large number of discharge filaments extending to the water surface, stabilizing at the multi-filament stage (the fourth stage). This discharge mode is capable of efficiently controlling the RNS and ROS concentration in the PAW, especially when the ROS/RNS ratios cannot be achieved by other discharge types.[290,291] It efficiently inhibits the growth and proliferation of human cancer cells due to the syngenetic effect of ROS and RNS in PAW.[291] Thus, discharge modes affect reactive species concentration as controlled through voltage and current, which plays an important role in PAW applications. The method to deliver plasma ROS/RNS into the water or transport ROS/RNS from plasma to water is summarized and illustrated in Figure 13b. The transfer of ROS/RNS largely occurs at the gas-liquid interface, involving many chemical and physical reactions such as redox reactions, photolysis, mass transfer, and particle collisions.[292] The whole process is as follows: ROS/RNS firstly generate in the gas phase plasma, and then transport to the plasma-water interface, and finally traverse the interface

and react with the molecules of the liquid phase. In aqueous solutions, ROS/RNS from plasma form numerous active species such as NO_2^-, H_2O_2, NO_3^-, ONOOH, and $ONOO^-$.[293] It is of utmost importance to understand the plasma-liquid interface to optimize and exploit new application areas.

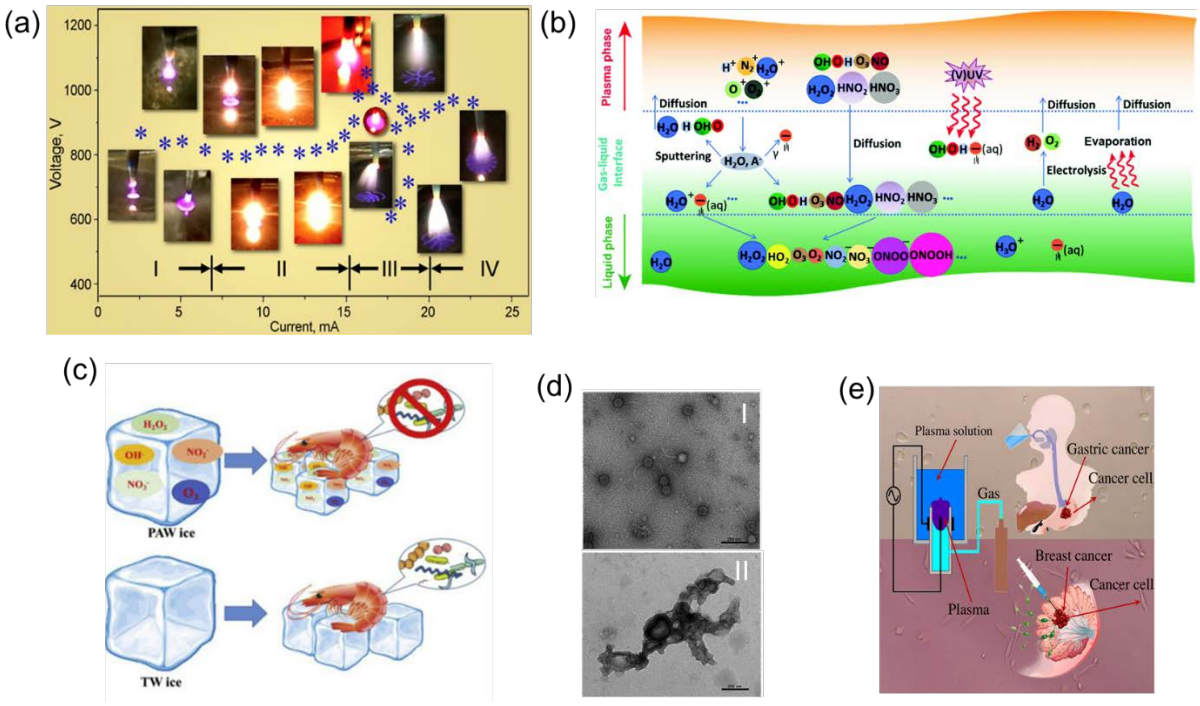

Figure 13. The generation mechanisms and biomedical applications of PAW. (a) Different discharge modes PAW with current-voltage dependence. Reproduced under the terms of the CC BY license.[294] Copyright 2017, Nature Publishing Group. (b) Important transfer processes at the interface of PAW. Reproduced from reference.[295] Copyright 2018, The Royal Society of Chemistry. (c) PAW efficiently inhibiting the microorganism's growth and retarding the loss of shrimp freshness and quality. Reproduced with permission.[296] Copyright 2018, Elsevier. (d) The T4 bacteriophages severely aggregating and forming large complexes after PAW treatment (I: control; II: PAW; scale bar: 200 nm). Reproduced under the terms of the CC BY license.[297] Copyright 2018, American Society for Microbiology. (e) Applications of PAW for gastric and breast cancer. Reproduced with permission.[288] Copyright 2016, Wiley-VCH.

Figure 13c shows PAW as a novel preservation method with high antimicrobial efficacy to maintain the safety and quality of food. PAW ice efficiently inhibits the microorganism's rapid growth and retards the loss of shrimp freshness and quality, exhibiting a longer shelf life. The effectiveness of PAW ice is owed to the abundant active species, in particularly stable ROS and RNS like H_2O_2, nitrate and ozone.[296] ROS in PAW ice oxidizes both intracellular components (such as proteins, DNA) and external cell walls to induce microbial inactivation.[136] Guo *et al.*

investigated the effect of PAW on T4 bacteriophages and indicated that PAW severely aggregates T4 bacteriophages (a typical structure with an icosahedron head and tail) to form large complexes compared with untreated, as shown in transmission electron microscopy (TEM) analysis of Figure 13d. The singlet oxygen in PAW plays a major role in the inactivation of bacteriophages through damaging both nucleic acids and proteins.[298,299] PAW provides a disinfecting strategy with convenience and no by-products, and prolonged storage has almost no effect on its antiviral activity. Figure 13e illustrates the applications of PAW for human gastric and breast cancer cells. ROS and RNS in PAW exhibit time-dependent behavior, and their synergistic effect plays a crucial role in the apoptosis rate.[291] PAW can be paired with drugs and swallowed to assist in fighting against cancers of the digestive system or injected into tumor areas for cancer therapy. Xu *et al*. studied the safety of PAW on immuno-deficient nude mice and revealed that PAW has no significant change in organ coefficient and tissue structure of the major organs including heart, kidneys, spleen, liver and lungs.[300] PAW with the same time treatment as in their previous results efficiently caused cancer cell apoptosis and reduced the bacteria viability.[209,301] PAW treatment has no unusual effects on the mental status, body weight, organ surface morphology, and organ coefficient. In addition, PAW has been applied for disinfecting oral pathogenic bacteria as a new type of mouthwash.[211] Moreover, a positive effect of PAW on seedling growth and plant germination rates has been reported, and PAW can also be applied as fertilizer for plants.[107] In summary, PAW appears to be a useful and powerful strategy for biomedical applications in food safety and other applications due to the presentation of ROS and RNS.

4.2 Plasma-activated saline solutions

Saline solution is on the World Health Organization's (WHO) list of essential medicines, the safest and most effective medicines needed in a health system. Plasma activation of saline solution generates chlorine/oxy-chlorine species, ROS, and RNS, all of which play crucial roles for biomedical applications.[302] Figure 14 shows the formation and biomedical applications of plasma-activated saline solutions (PASS). In PASS, different reactive chlorine/oxy-chlorine species (RCS) are generated in addition to the generation of ROS/RNS (as shown in Figure 14a).[209] Cl^- is the precursor for all the RCS and initially reacts with •OH, O_3, NO_3, and N_2O_5 to generate the first RCS (ClO^-, $ClNO_2$, and Cl). Hypochlorite (ClO^-/HClO) is the dominant RCS and drives the transformation chain for RCS: $Cl^-/Cl_2^- \rightarrow ClO^- \rightarrow ClO_2^- \rightarrow ClO_2 \rightarrow ClO_3$.[303] Comparing with PAW,

the concentration of ROS/RNS is lower due to generation of RCS consuming a large amount of ROS/RNS; its bactericidal effect is much higher, however, because RCS play an important role in disinfection.[209] Griseti *et al*. evaluated the cytotoxic effect of CAP-activated phosphate-buffered saline (PBS) on multicellular tumor spheroids (MCTs) cells.[304] The ultrastructure of MCTs cells through TEM after treatment of plasma-activated PBS was assessed to indicate large and swollen nuclei with either nuclear fragmentation (karyorrhexis) or a condensed nucleolus (pyknosis) in Figure 14b. The combination of plasma-activated PBS and electropermeabilization induction altered the cell-cell junctions within the MCTS and higher plasma-activated PBS penetration.[305-307] The combined treatment caused the caspases activation and an earlier onset of DNA damage, completely abolishing MCTs growth. Furthermore, Joshi *et al*. explored genome-wide response in Escherichia coli treated by plasma-activated PBS through DNA microarray and demonstrated plasma-activated PBS conversely downregulating genes related to energy generation, housekeeping and metabolism, motility and virulence.[308] Genes responsive to ROS/RNS/RCS in plasma-activated PBS are emerging as playing an important role in cellular stress regulation.

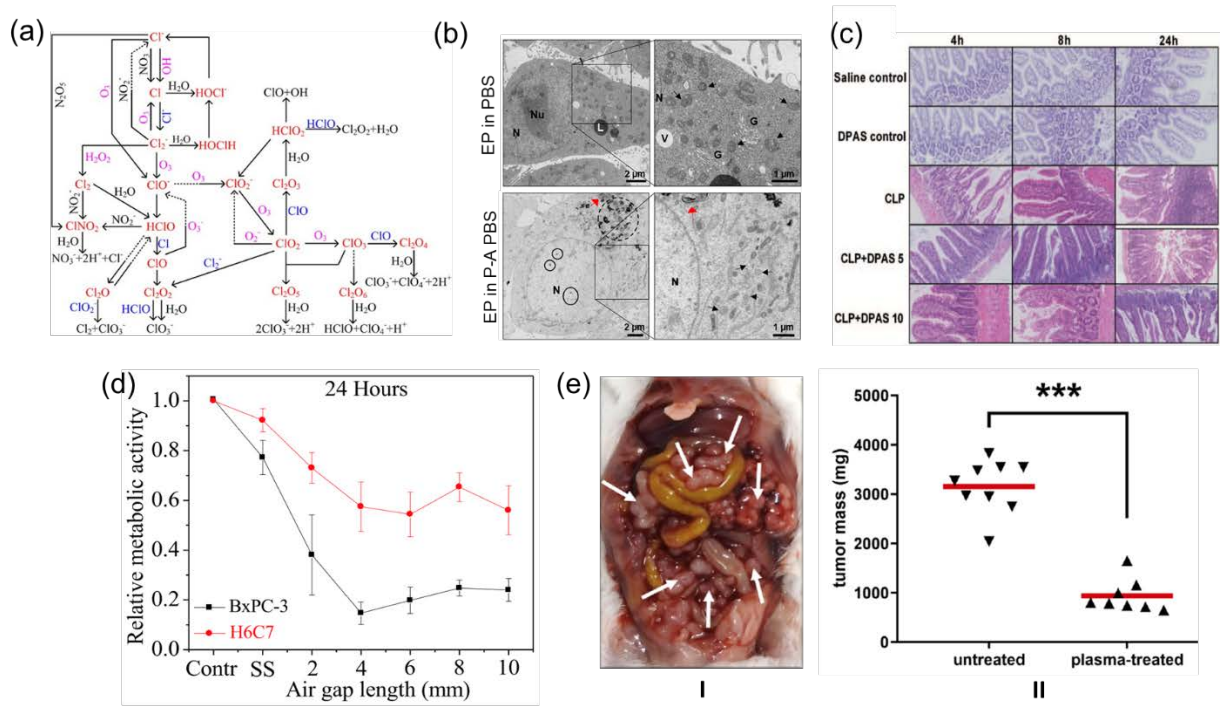

Figure 14. The formation and biomedical applications of plasma-activated saline solutions (PASS). (a) Potential chemical profile of reactive chlorine/oxychlorine species in PASS. Reproduced from reference.[209] Copyright 2017, Wiley-VCH. (b) Ultrastructure modifications of the multicellular tumor spheroids after plasma-activated phosphate-buffered saline (PBS) treatment (EP: electropermeabilization; Nu: nucleolus;

N: nucleus; V: vacuole; L: lysosome; G: Golgi apparatus; black arrows: mitochondria; red arrow: lamellar bodies, autophagic; black dashed-line circles: vacuoles; black solid-line circles: pyknosis/karyorrhexis). Reproduced under the terms of the CC BY license.[304] Copyright 2019, Nature Publishing Group. (c) Discharge plasma-activated saline (DPAS) treatment alleviated cecal ligation and puncture (CLP)-induced abdominal sepsis in mice (H&E at 4, 8, and 24 h; magnification 200×) (low or high doses of DPAS 5 mL/kg or 10 mL/kg). Reproduced from reference.[309] Copyright 2019, Shock Society. (d) PASS manifesting selective treatment on human pancreatic cancer cells. Reproduced with permission.[302] Copyright 2018, IEEE. (e) Intraperitoneal burden of CT26 colon cancer cells in Balb/C mice (I) and tumor weight after plasma-treated saline treatment (II). Reproduced under the terms of the CC BY license.[310] Copyright 2019, Nature Publishing Group.

Zhang *et al*. studied the effect of the discharge plasma-activated saline (DPAS) on abdominal sepsis caused by cecal ligation and puncture (CLP), and their intestinal tissues were collected and stained with H&E as shown in Figure 14c. H&E staining exhibited severe hemorrhage and congestion, surface ulceration, inflammatory cell infiltration, edema, and atrophic/shortened mucosal villi in the CLP group, while DPAS obviously reversed these impaired pathological changes.[309] Due to the presentation of ROS, RNS, and RCS, DPAS treatment remarkably attenuated NF-kB p65 and high-mobility-group-protein B1 (*HMGB1*) expression levels, inflammatory reaction, lung wet/dry ratio, bacterial load in the peritoneal blood/cavity/lungs, the lung/intestine histopathological injuries, and cell apoptosis in the lung/intestine.[311,312] Figure 14d shows selective killing by PASS on pancreatic cancer cells, and PASS had a stronger effect on BxPC-3 cancer cells than H6c7 normal cells. Intracellular ROS -mediated up-regulation of DR5 was able to cause apoptosis in BxPC-3 cancer cells, and intracellular generation of ROS led to increased protein expression of Bax and disruption of the mitochondrial membrane potential.[313,314] In addition, PASS may affect non-oncogene dependencies in the transformed genotype context and induce selective killing through a synthetic lethal interaction.[315] Freund *et al*. indicated that PASS promoted an immunogenic phenotype of CT26 colon cancer cells *in vivo* and *in vitro*, and their results are shown in Figure 14e. PASS treatment resulted in a significant upregulation of CRT on CT26 colon cancer cells for positive therapeutic outcomes in tumor-patients. CRT is a leading marker for ICD and a chaperon derived from ER translocating to the cell surface to play the role as “eat-me” signal for tumor phagocytosis.[316,317] Further, other damage-associated molecular patterns (DAMPs) are also observed, such as *HMGB1* and heat-shock protein 70 (*HSP70*)

increasing on CT26 colon cancer cells surface after PASS treatment. *HSP70*, a chemoattractant for neutrophil granulocytes and monocytes, induced maturation of DCs.[318] *HMGB1* can bind to many receptors such as *TLR4*, *TLR2*, *Tim3*, and *RAGE*, which indicates that PASS has the potential to activate antitumor immunity.[319]

PASS has shown great potential in biomedical applications, especially for cancer therapy. Additionally, it also has the potential to be utilized as either an oral medicine or as a drug which can be injected into blood. As presented in the 2017 Plasma Roadmap, plasma-activated liquid is an important new and novel development still challenged by limited studies on the underlying mechanisms.

4.3 CAP-activated other media

Besides the plasma-activated water and saline solutions mentioned above, scientists also employ plasma to activate culture medium (such as DMEM (Dulbecco's Modified Eagle Media) and RPMI (Roswell Park Memorial Institute)), Ringer's lactate solution, and N-Acetylcysteine (NAC) solution.[320,321] Culture medium is used to prepare plasma-activated media (PAM) consisting of many types of amino acids, inorganic salts, and vitamins. The efficiency of PAM is due to the generation of ROS and RNS after exposure of media to plasma. Figure 15 shows the biomedical applications of PAM. Nakamura *et al*. revealed that plasma-activated media (RPMI-1640) suppressed the metastatic potential of ovarian cancer, and their mouse model is shown in Figure 15a. Matrix metalloproteinases, such as *MMP-9*, are known to digest the extracellular matrix (ECM), which is an essential step in cancer cell metastasis and invasion.[322] ROS in PAM inhibits cancer cell invasion and migration *via* blocking the *MMP-9* pathways.[323] PAM prohibits the activation of the MARK pathway through preventing the phosphorylation of *p38* MAPK and *JNK1/2* to decrease the expression of *MMP-9*, which is critical for cancer cell motility.[324]

Figure 15b indicates PAM (Stemfit AK03 medium) selectively eliminating undifferentiated human induced pluripotent stem cells (hiPSCs). A proportion of hiPSCs treated by 4-fold diluted PAM appear shrunken and are positively stained by PI. The reason for the higher sensitivity of undifferentiated hiPSCs induced by PAM might be that hiPSCs have lower oxidative stress-related gene (CAT, GPX1, and ATM) expression levels than normal cells.[325] In addition, media containing

the same concentration of H_2O_2 kills undifferentiated hiPSCs less efficiently than PAM, suggesting the synergistic effect of H_2O_2 and other reactive species in PAM selectively killing undifferentiated hiPSCs.[326] Plasma-activated culture media has been intensively investigated and demonstrated its significant antitumor capacity on dozens of tumors.[320,327]

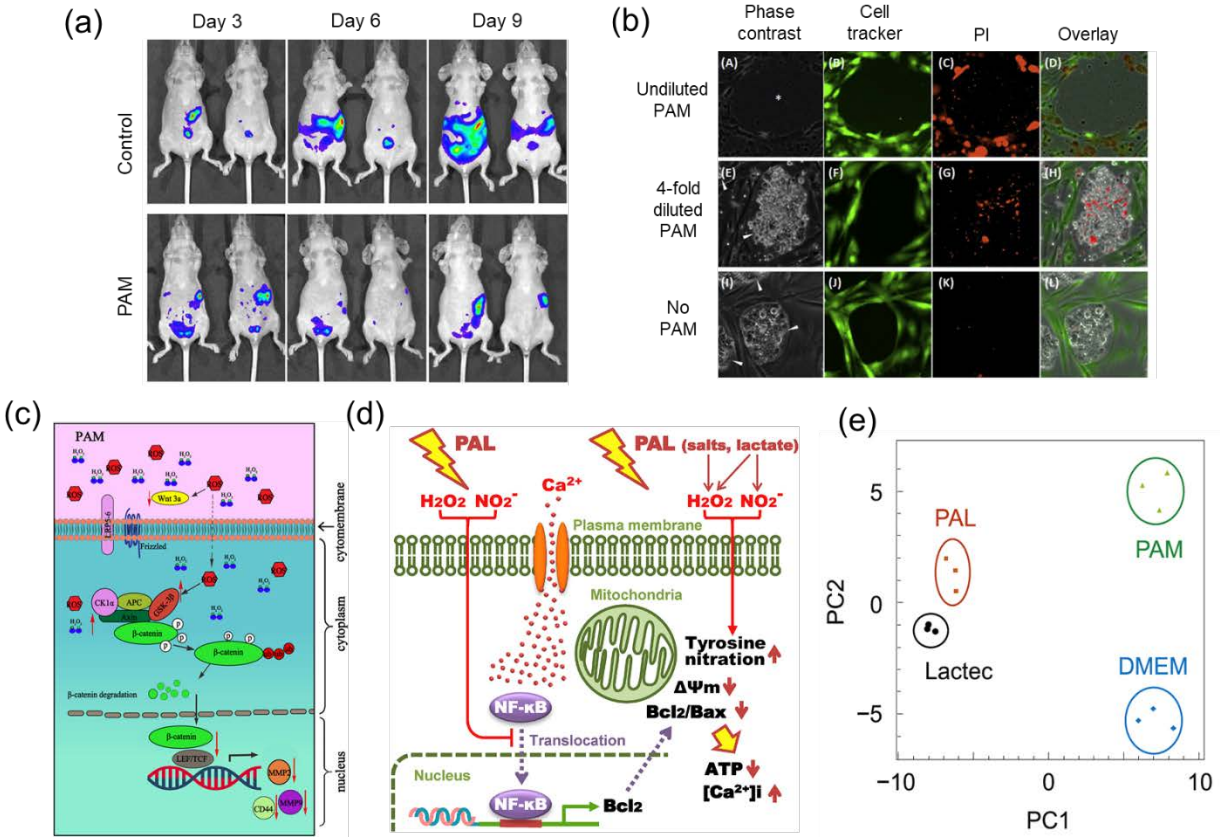

Figure 15. The biomedical applications of PAM. (a) PAM suppresses intraperitoneal metastasis in an ovarian cancer mouse model. Reproduced under the terms of the CC BY license.[323] Copyright 2017, Nature Publishing Group. (b) PAM selectively eliminates undifferentiated 201B7 hiPSCs and differentiated cells NHDFs (Asterisk and arrow are hiPSCs before and after PAM treatment, respectively). Reproduced with permission.[326] Copyright 2016, Elsevier. (c) Schematic of plasma-activated medium (RPMI-1640) suppressing the canonical Wnt/β-catenin pathway. Reproduced with permission.[328] Copyright 2020, Wiley-VCH. (d) Plasma-activated lactated Ringer's solution (PAL) induces A549 cell injury. Reproduced with permission.[329] Copyright 2018, Elsevier. (e) Plot of principal component analysis (PCA) scores for PAL-treated cells in comparison with PAM (DMEM). Reproduced with permission.[330] Copyright 2020, Elsevier.

Liu *et al*. examined the mechanism of PAM (RPMI-1640) inhibiting metastasis and invasion of B16 melanoma cells and indicated PAM suppressed the *Wnt/β-catenin* pathway shown in Figure

15c. Generally, PAM displays a time-dependent behavior on the inhibition of invasion and migration effects on cancer cells. ROS/RNS in PAM plays a role in anticancer activity through DNA damage, cell apoptosis, and cell injury.[331] ROS can degrade *β-catenin* and prevent *TCF/β-catenin* transcriptional activity through activation of the forkhead box-O transcription factor.[332] Moreover, H_2O_2 can cause *GSK-3* activation and rapid dephosphorylation, losing *β-catenin* nuclear location and reducing *β-catenin* protein.[333] Also, H_2O_2 can degrade *β-catenin via* stabling the axin, *APC*, *DVL*, and *GSK-3β*.[334] In addition, PAM-treated B16 cells change the metastasis and invasion through metastasis- and invasion-related proteins such as *MMP-9*, *MMP-2*, *E-cadherin*, and *CD44* (see Figure 15c).[328]

Lactated Ringers' solution consists of only 4 components. Figure 15d manifests signaling mechanism for plasma-activated lactated Ringers' solution (PAL) induced A549 cancer cell injury. The PAL was significantly stronger in suppressing A549 cell viability than PAM due to components in culture medium weakening the anti-tumor activity of PAM by scavenging non-specific ROS/RNS.[335] PAL generates significant amounts of nitrite and H_2O_2, and nitrite synergistically increases H_2O_2-induced cell injury.[336] PAL treatment inducing reductions in $\Delta\psi m$ and tyrosine nitration results in mitochondrial dysfunction with *NF-κB-Bcl2* signaling downregulation.[337] These reactions might cause the elevations in $[Ca^{2+}]i$ and ATP depletion and apoptosis ultimately.[329] In addition, Bisag *et al*. illustrated that PAL can be used as novel clinical therapy with a local administration acting on killing cancer cells with no or minimal damage on the surrounding healthy tissues.[338] Ishikawa *et al*. compared PAM and PAL induced changes in metabolomic profiles in cells and plotted their principal component analysis (PCA) scores in Figure 15e. The PCA score plots demonstrate 20.3% and 47.0% of the variation explained by PC2 and PC1, respectively. For PC1, metabolites with the highest absolute factor are predominantly related to lipid metabolism and amino acid; for PC2, those are primarily involved in the pentose phosphate and glycolysis pathway.[330] U251SP cells treated by PAL display changes in intracellular metabolites being reductive in the redox state, while PAM exhibits the oxidative stress condition.[339] Acetyl-CoA generation increases for lipid metabolism from asparagine and alanine in the metabolomic profiles of PAL-activated cells.[340] Therefore, PAL causes regulated death to glioblastoma cells in more innate microenvironments than the PAM. Besides PAM and PAL,

authors also apply plasma to activate N-acetylcysteine solution for bacterial inactivation.[341] PAM provides the indirect treatment of plasma as safe plasma medicine.

5. Biomedical device-assisted plasma delivery

The applications of most plasma systems are often restricted owing to factors like regions with difficult access and internal organs treatments. Plasma efficacy remains unsatisfactory in some situations due to limitations on the penetration of CAP to tissues, requiring diverse delivery methods to achieve desirable therapeutic effects. A lot of effort has been made to deliver plasma-generated reactive species far from sources, where a localized or internal treatment can be achieved.[342] In addition, the demand for convenient, small, and flexible devices to be handled has been growing exponentially over recent years. Therefore, delivering plasma through biomedical devices has received a lot of attention for both fundamental physics and practical biomedical applications.

5.1 Small tubes for delivery of plasma

CAP delivery by most conventional devices is not amendable to biomedical applications inside the living organism due to drawbacks such as high voltage, plasma probe volume, and gas delivery. Plasma devices using small tubes can address this challenge by delivering reactive species directly to the target for biomedical applications.[343,344] Figure 16a shows He plasma jet can be delivered 130 cm away after ignition by sawtooth waveform with a 4 kV peak-to-peak potential. This plasma tube is electrically safe and can be handled with bare hands for biomedical applications. The higher applied voltage and lower diameter benefit the longer distance of plasma delivery and higher reactive species density. Other studies also indicate the distance of plasma delivery in tubes depending on plasma sources and tubes' length/thickness.[342,345,346] For example, He plasma can be delivered in a plastic tube with a length up to 200 cm, while Ar plasma can be delivered only at a much shorter distance. The radial and longitudinal components of the electrical field for He plasma remain nearly constant over tens of centimeters inside a dielectric tube of 70 cm long and 0.4-1.6 cm inner diameter.[347]

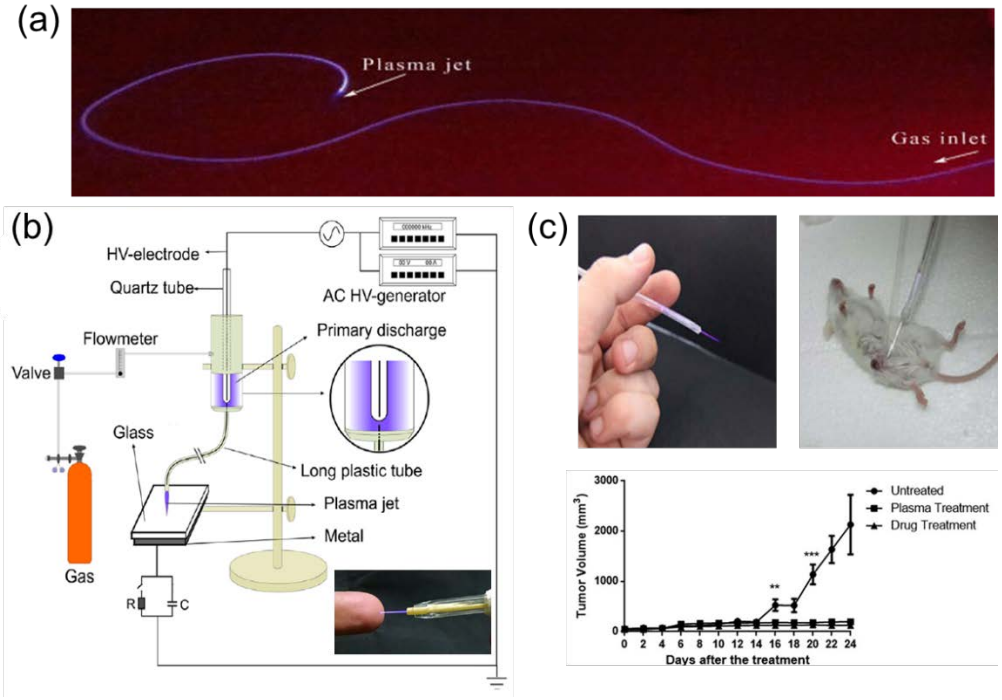

Figure 16. Small tubes help to delivery and extend plasma biomedical applications. (a) Plasma delivery using a single electrode configuration through a sub-millimeter flexible dielectric tube beyond 130 cm. Reproduced with permission.[348] Copyright 2014, AIP Publishing LLC. (b) Experimental setup for plasma jet emerging from the end of 100 cm long plastic tube and directed on a finger. Reproduced with permission.[349] Copyright 2015 Wiley-VCH. (c) Plasma delivery through a small tube to the animal body for the tumor treatment application including *in vitro* and *in vivo* analysis. Reproduced under the terms of the CC BY license.[350] Copyright 2016, Nature Publishing Group.

Figure 16b exhibits an experimental setup for plasma delivery over 100 cm length in a small tube whose anti-microbial efficiency was tested against fungus Candida albicans. The plastic tube delivering plasma jet from the high-voltage discharge region over 100 cm in length does not change the plasma composition.[349] The excited N_2 molecules are primarily generated from electron impact reactions due to the plasma plume propagating in the ambient air. The fungi were killed by the long-distance delivered plasma, and its mechanism of cell destruction is similar to plasma jet due to the same reactive species generated in plasma plume.[19,117] Therefore, the flexible plastic tube works as an extension pipe for *in vitro* microbial decontamination, which is able to be easily directed and bent to a target with same effect as the stationary plasma jet. Mirpour *et al*. applied a small tube to assist in plasma delivery for tumor treatment shown in Figure 16c. Flow cytometry

results indicated more than 80% cancer cells undergoing apoptosis after plasma radiation assisted by a small tube. The tumor size after plasma treatment is also shown in Figure 16c, and the tumor suppression induced by plasma significantly differs from the untreated group after 16 days. Use of a small tube to assist with plasma delivery leads to the anti-oxidative mechanisms in the cells resulting into cell death.[103] Although disadvantages (such as side effects, high cost, and low rapidity) exist, conventional methods, including radiotherapy, surgery, and chemotherapy, are now still being in the use for cancer therapy.[272,351,352] While small-tube assisted plasma delivery may overcome the adverse effects of the conventional treatments, it should be noted that plasma delivery assisted by small tubes with great flexibility can be directed to places or organs difficult to access for clinical applications.

5.2 CAP delivery through micro-sized tube

For biomedical applications involving resistance to conventional therapies, susceptibility to damage with conventional therapies, and very limited capacity, scientists developed micro-sized needles for CAP delivery, as shown in Figure 17. Figure 17a represents plasma delivery through micro-sized stainless-steel tubes. The total electron number from a micro-sized tube with 20 mm and 60 mm length is 4.60×10^{12} and 4.04×10^{12} for one discharge period, respectively, and the 20 mm length generates higher concentrations of O_2^-, •OH, H_2O_2, NO_2^- than 60 mm. Although plasma delivery with 20 mm length micro-sized tube has a stronger antitumor effect, 60 mm length microneedles can still efficiently kill cancer cells due to the synergetic effects of reactive species and free radicals.[353] For dentistry, bacteria and remnants on pulp tissues might induce irreversible damage of tooth pulp.[354] Bacteria residing in the side canals or in the apical root canal delta are difficult to access *via* rinsing or flushing with disinfectants.[355] Bussiahn *et al*. developed a plasma filament with the diameter of 30 μm and a length of up to 15 mm which can help to overcome the above problems with endodontic treatment in dentistry. This hairline plasma has been applied to the prepared root canal of a human tooth in a grounded bulk of the meat (as shown in Figure 17b) and killed these residual bacteria.[356] In addition, this 30 μm hairline plasma can be applied to treat the human skin or organ without any painful irritation or heating. Chen *et al*. developed a diameter of 70 μm plasma jet, which was first applied to the glioblastoma in mice brains (shown in Figure 17c). The glioblastoma tumor in the control group increased approximately 600% over two days, whereas 70 μm plasma jet treated tumor volume decreased nearly 50% compared with baseline

levels. This intracranial micro-sized plasma jet not only prevents glioblastoma tumor growth in the mouse brain, but also reduce tumor size.[357] In addition, this micro-sized plasma jet can circumvent four major limitations of glioblastoma tumor treatment and recurrence: (1) brain has a limited capacity, (2) brain is susceptible to damage, (3) tumor cells have resistance, and (4) blood-brain barrier stop drugs entering brain.[358,359] Plasma delivery through micro-sized tubes is a milestone for plasma medicine, which can be clinically applied to tumors or organs in human body.

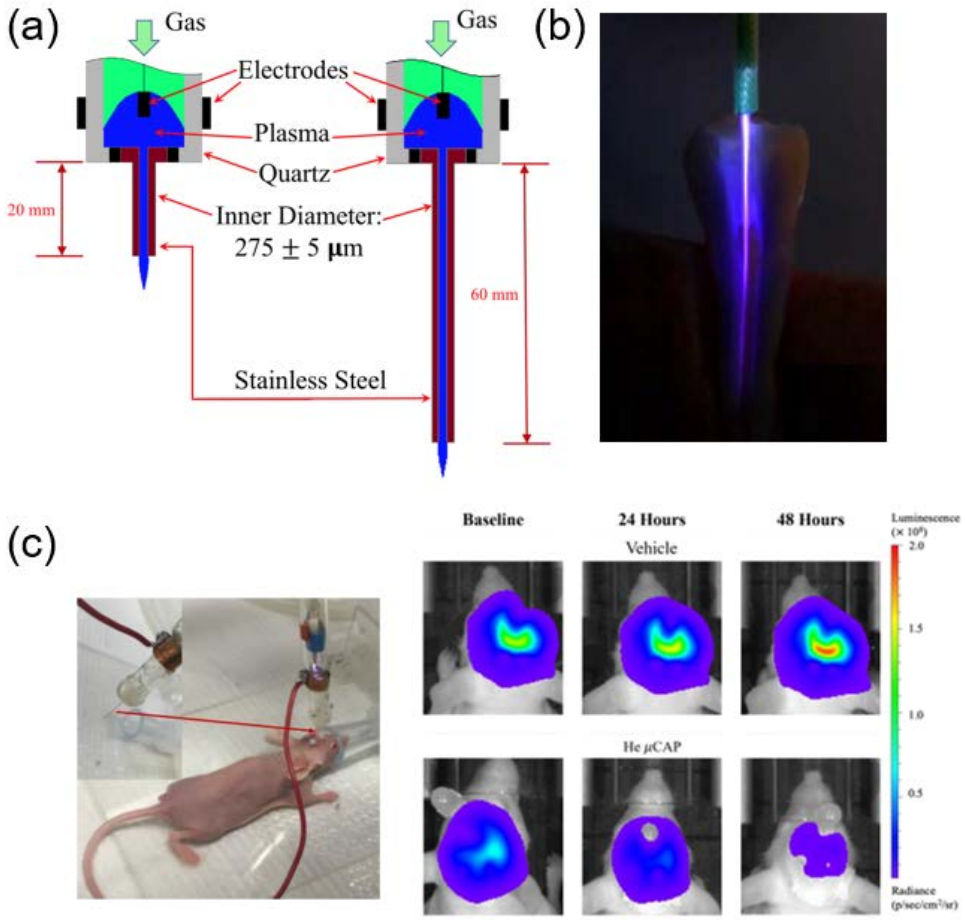

Figure 17. CAP delivery through micro-sized tube. (a) Plasma delivery through micro-sized tube (275 ± 5 μm inner diameter) with different length applied to cancer cells. Reproduced with permission.[360] Copyright 2018, Begell House Inc. (b) Micro-sized tube (30 μm) assisting plasma delivery to a prepared root canal of a human tooth in a grounded bulk of the meat. Reproduced with permission.[356] Copyright 2010, AIP Publishing LLC. (c) Plasma delivery *via* micro-sized tube (70 ± 3 μm inner diameter) applied to glioblastoma both *in vitro* and *in vivo*. Reproduced under the terms of the CC BY license.[357] Copyright 2017, MDPI, Switzerland.

5.3 CAP delivery *via* patch

Microneedle-array patch-mediated transdermal drug delivery has made a paramount contribution to the simplicity of therapeutics administration. It has been proved that microneedle patches consisting of miniature needles can easily perforate the stratum corneum and effectively transfer drugs into the dermis in a minimally-invasive and painless manner. While the main efforts of using microneedles have been concentrated on the delivery of synthetic drugs, biomacromolecules, or even drug-nanoformulations, our recent report using a microneedle-array path with a unique structure represents an important advance for the field of plasma medicine. CAP is known to have limited tissue penetration. To overcome this challenge for CAP therapy, a hollow-structured microneedle patch (hMN) was designed (Figure 18), where each hollow-structure microneedle serves as microchannels to allow CAP to be delivered through the skin into the tumor tissue (i.e., melanoma). Results indicate that a significant amount of reactive species in CAP penetrated through the hMN compared to the results when replaced with a microneedle patch that has no hollow structures (Figure 18). Moreover, once applied on the mouse skin, the hMN could facilitate the delivery of CAP through the skin with an efficiency significantly higher than CAP directly through bare skin tissue or the skin applied with a solid microneedle patch. In a melanoma mouse model, the CAP/hMN device significantly induced an enhanced anti-tumor effect compared with direct CAP application on the skin or CAP through a solid MN patch. All these data suggested a beneficial role of hollow-structured MN in delivering CAP. This delivery platform has also been combined with immunotherapeutics (i.e., immune checkpoint inhibitors) to further enhance the antitumor efficacy, which will be elaborated in the later section. It is noted that the hollow-structured microneedle was made of highly biocompatible polymers, namely polyvinylpyrrolidone (PVP) and polyvinyl alcohol (PVA), which generated minimal inflammation in the skin tissue.

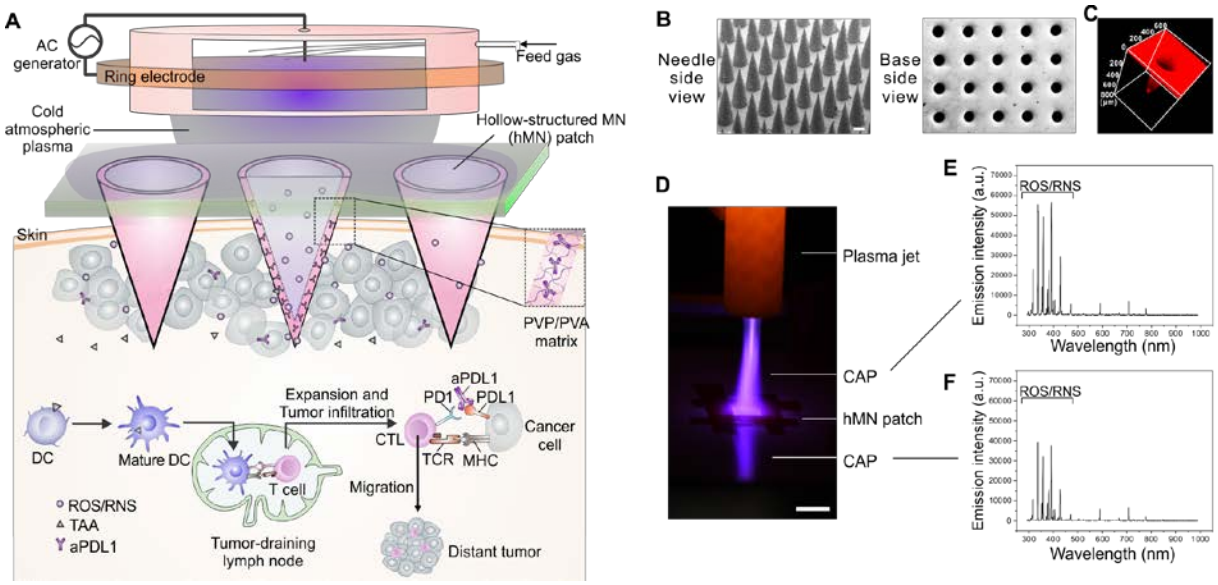

Figure 18. (a) Illustration of the transdermal CAP-mediated immune checkpoint blockade (ICB) therapy. Schematic of the transdermal combination of CAP and ICB therapy assisted by the polymeric hollow-structured microneedle patch loaded with aPDL1. Nomenclature: DC, dendritic cell; TAA, tumor-associated antigen; CTL, cytotoxic T lymphocyte; TCR, T cell receptor; MHC, major histocompatibility complex; PVP, polyvinylpyrrolidone; PVA, polyvinyl alcohol; ROS, reactive oxygen species; RNS, reactive nitrogen species; AC, alternating current. (b) Representative SEM images of the hMN patch from views of needle side and base side. (c) A 3D CLSM image of hMN patch (rhodamine loaded). (d) Penetration test of the CAP through hMN patch. (Scale bar, 1 cm.) The CAP through the hMN included reactive species and reflection. Representative OES spectra of the CAP (e) above hMN patch and f) penetrating through the hMN patch. Reproduced with permission.[361] Copyright 2020, National Academy of Sciences.

6. Combination of CAP delivery and other therapeutics

Due to CAP being composed of various reactive species, it is capable of consistently producing different pharmaceuticals on demand. CAP can adaptably combine with other drugs used as oral medication or injected into blood. This section will summarize the recent progress in CAP delivery with drugs and discuss their benefits and challenges.

6.1 CAP delivery with nanoparticles

The synergistic combination of CAP technology and nanoparticle delivery systems has shown the potential in biomedical applications.[362] This combination is likely due to the enhanced selective permeability of plasma species by inducing membrane rupture, leading to the intracellular diffusion of nanoparticles to disease site in the tissue. For example, gold nanoparticles (AuNPs) combined with CAP can markedly promote the death of cancer cells. AuNPs can be employed as drug delivery vehicles, radiosensitizers, and diagnostic agents because of their specific chemical and physical properties, such as low cytotoxicity, high stability, and strong surface plasmon resonance effect.[363,364] Generally, AuNPs are considered nontoxic to normal cells, while non-functionalized AuNPs exhibit selective cytotoxicity to certain cancer cell lines. He *et al*. investigated synergistic mechanisms of DBD and AuNP induced cytotoxicity in cancer cells.[365] Figure 19a presents AuNP (red) inside lysosomes (green) confirmed by rotating and sectioning the cells around the three spatial axes. Plasma induces synergistic cytotoxicity and stimulates the citrate capped 20 nm AuNP uptake through the predominantly endocytic mechanism at the same time, resulting in the trafficking of AuNP into acidic (lysosomal) compartments of U373MG cells. Kaushik *et al*. reported that co-treatment of plasma and PEG-coated AuNPs inhibited the proliferation of cancer cells by eliminating the activation of PI3K/AKT signal axis.[366] Co-treatment with plasma and GNP leads to cellular and nuclear changes in solid tumor cells. TEM visualizes nuclei and mitochondrial ultrastructure of T98G cells with co-treatment (Figure 19b). Co-treatment reversed EMT in solid tumor cells by reducing the secretion of a large number of proteins, leading to the upregulation of epithelial markers. The additional recent result indicated that strong synergy exists between CAP and AuNPs in cancer therapy.[367-369] The synergistic combination of CAP technology and nanoparticle delivery systems also correlate well with the theory that intracellular RNS/ROS accumulation induces oxidative stress and further changes the intracellular pathways, leading to damage to the proteins, lipids, and DNA.

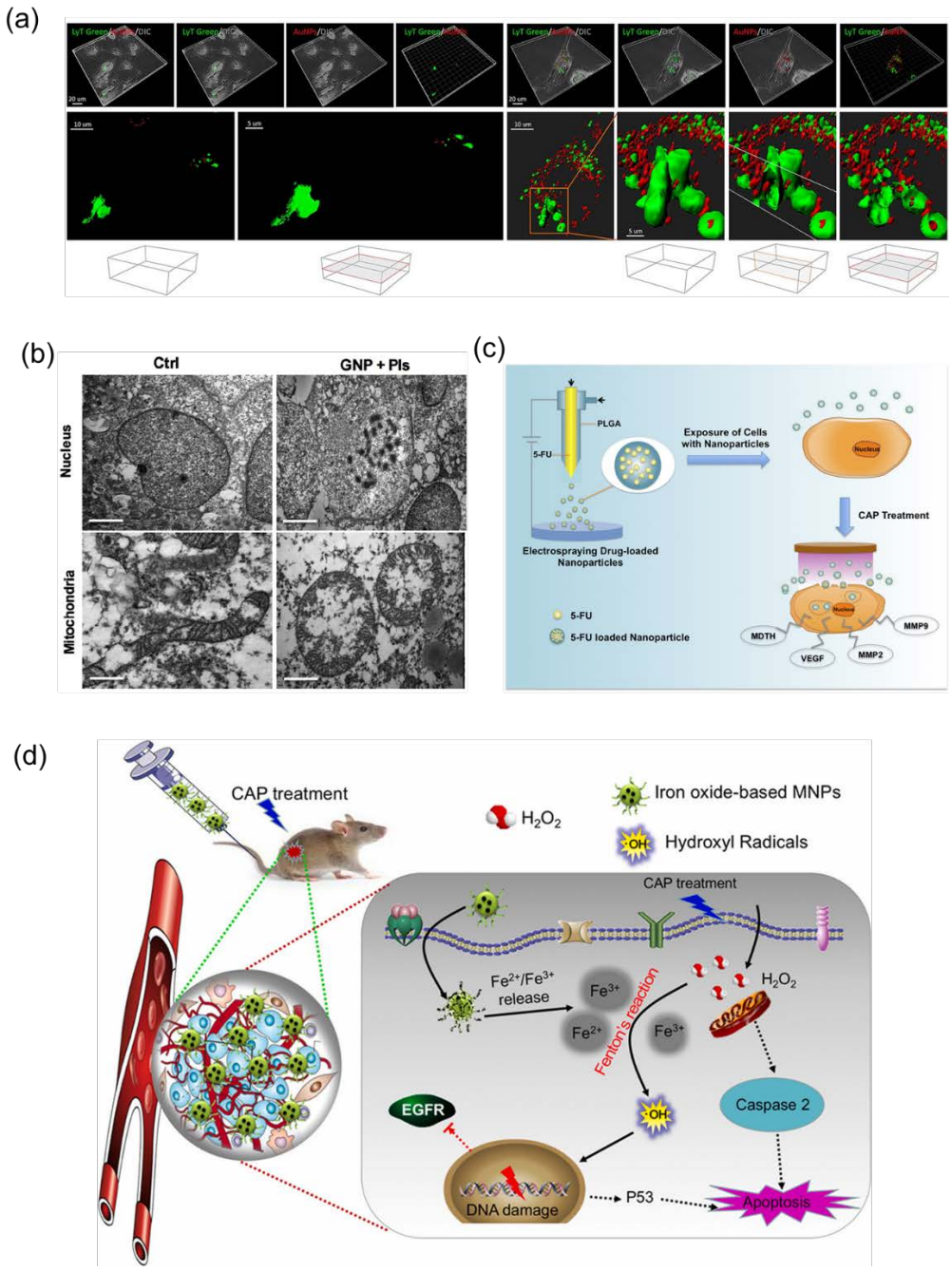

Figure 19. Plasma delivery with nanoparticles. (a) AuNPs are incorporated within lysosomes in glioma cells. AuNP (red) inside lysosomes (green) confirmed by rotating and sectioning the cells around the three spatial axes. Reproduced under the terms of the CC BY license.[365] Copyright 2018, Nature Publishing Group. (b) TEM for T98G cells co-treated with GNP and plasma to visualize nuclei and mitochondrial ultrastructure. Reproduced with permission.[366] Copyright 2016, Elsevier. (c) Electrosprayed core-shell nanoparticle fabrication and CAP facilitated drug delivery. Reproduced under the terms of the CC BY license.[370] Copyright 2016, Nature Publishing Group. (d) Molecular mechanisms of magnetic nanoparticles

(MNPs) enhancing tumor-selective killing effect of plasma. Reproduced with permission.[371] Copyright 2019, Elsevier.

Nano-based drug delivery devices have revolutionized cancer treatment due to effective and sustained targeted delivery of therapeutic agents to solid tumors.[372] Zhu *et al*. developed a new synergistic targeted breast cancer therapeutic method of novel drug loaded core-shell nanoparticles and plasma (Figure 19c).[370] Compared to each treatment separately, the co-treatment of plasma and drug loaded nanoparticles exhibited much better effectiveness for inhibiting the cell growth of breast cancer. Plasma causes downregulation of metastasis involved gene expression (*MTDH*, *VEGF*, *MMP2*, and *MMP9*) as well as facilitated drug loaded nanoparticles uptake what may be promoted to minimize drug resistance-a main problem in chemotherapy. Li *et al*. developed a novel co-treatment of plasma and iron oxide-based magnetic nanoparticles (MNPs) for targeted lung cancer treatment.[371] The synergistic effectiveness of plasma and iron oxide-based MNPs strongly killed lung cancer cells and seriously inhibited cell proliferation by inducing apoptosis and reducing viability. Plasma-originated RONS will induce a noticeable rise of intracellular H_2O_2, meanwhile Fe^{2+}/Fe^{3+} released from the lysosome could catalyze H_2O_2 into •OH, which results in the injury of cancer cells, such as inducing double strand DNA breaks and mitochondria-mediated apoptosis (Figure 19d). Jalili *et al*. also indicated that the combination of CAP and iron nanoparticles significantly decreased the viability of cancer cells and induced shifting of the *BAX/BCL-2* ratio in favor of apoptosis.[373] Ouf *et al*. applied CAP to mediate silver nanoparticles (AgNPs) against dermatophyte fungi.[374] AgNPs in conjunction with CAP have advantages over conventional and currently used azole compounds, because they have a good safety profile and limited side effects, especially when employed topically to treat superficial mycotic infections.[375,376] Moghanloo *et al*. indicated that seed priming with plasma and silica nanoparticles provoked molecular, physiological, and anatomical changes, thereby strengthen the growth and protection of plants.[377] Combining plasma advantage with nanoparticles opens up multiple benefits such as enhancing plasma action and nanoparticle uptake outlined above. In addition, employing this strategy can lead to a reduction of overall toxicity.

6.2 CAP with immunotherapy

Cancer immunotherapy has gained increasing popularity and momentum in recent years.[378,379] The Nobel Prize in Physiology or Medicine 2018 was awarded to Allison and Honjo, for their historic work on immune checkpoint blockade (ICB).[380,381] Cancer immunotherapy aims to elicit or strengthen anti-tumor immunity to fight against tumor cells. Particularly, ICD is a form of programmed cell death that has the potential to initiate and stimulate tumor-specific adaptive immune responses.[318,382] Many chemical and physical methods have been investigated to elicit ICD for enhanced cancer immunotherapy, including certain chemotherapeutics (e.g., doxorubicin), phototherapy, and radiation therapy.[383] When ICD occurs, immunostimulatory molecules, collectively called DAMPs, are released from or displayed by the dying cells, which could be rapidly recognized by innate immune cells (e.g., macrophages and dendritic cells) and then further activate adaptive immune responses (T cell-mediated).[318,384]

More recently, the synergistic actions of ROS and RNS in CAP have also been demonstrated to induce cancer ICD in various cancer cells.[385] Specifically, ROS/RNS can elevate the oxidative stress in tumor cells after CAP treatment, which leads to the release of critical *DAMPs*, including surface-expressed calreticulin (*ecto-CRT*), ATP, *HMGB1*, *HSP70*, and *HSP90*.[316,386-388] These molecules are performing important roles for maintaining cellular integrity and function, but they become immunogenic outside the cell (e.g. membrane-bound or released). Once externalized, *DAMPs* initiate various immunologic responses by attracting innate immune cells (e.g., dendritic cells and macrophages) to the tumor tissue. ATP and *HMGB1* act as 'find me' signals and chemotactically recruit antigen-presenting cells (APCs), including macrophages and DCs, to the area of *DAMP* emission. *Ecto-CRT* acts as an 'eat me' signal, promoting phagocytosis of *DAMP*-releasing cells by APCs and activating APCs.[389] The activated APCs then present tumor-associated antigen to T cells in the lymphatic organs, resulting in the generation and expansion of tumor-specific effector and memory T cells.[390] Recently, it has been discovered that CAP treatment could induce the higher expression of ICD markers in several tumor types, including melanoma, colorectal tumor cells, pancreatic cancer cells, and leukemia cells.[361,391-394] Interestingly, it is also found that cancer cells culturing in CAP-pre-treated media (indirect CAP treatment) can also undergo ICD.[395]

The releasing tumor-associated antigens during CAP-mediated ICD can promote DC maturation to the T cells and activate T cell priming.[361] Chen *et al*. reported that, after transdermal CAP treatment on melanoma syngenetic mice, an increased population of mature DCs was observed after CAP treatment on tumors. Furthermore, increased numbers of infiltrating T lymphocytes (*CTLs, $CD3^+$*) have been identified, accompanied by the elevated fractions of effector T cells (*$CD3^+CD4^+$*) and cytotoxic T cells (*$CD3^+CD8^+$*), indicating the enhanced tumor-specific T-cell-mediated immune responses *via* CAP treatment. In combination with immune checkpoint blockade therapy, anti-programmed cell death-1 ligand 1 antibody (*aPDL-1*), which can block PD/PDL1 pathways between T and tumor cells, further augmented T-cell-mediated antitumor efficacy. This represents the first demonstration of the synergy between CAP therapy and ICB therapy.[361] Figure 20 shows CAP with aPDL1/CAP could trigger a systemic immune response against distant tumors. The tumor on the right flank as the primary tumor was treated with CAP/aPDL1, while the distant tumor on the opposite site received no treatment to mimic distant tumors (Figure 20a). The bioluminescence signal from the tumors and size of the tumors in the mice treated with CAP/aPDL1 significantly decreased compared with untreated controls (Figure 20c and d). More importantly, compared with untreated mice, the distant tumors (left tumors) of the treated mice have also been inhibited effectively. Consistently, the weights of primary and distant tumors in the treatment group were also lower than those in the untreated group (Figure 20d). Lin *et al*. also report that CAP treatment on CT26 colon carcinoma cells induced ICD and elicited tumor-specific immunity and can act as an adjuvant for cancer therapy.[391]

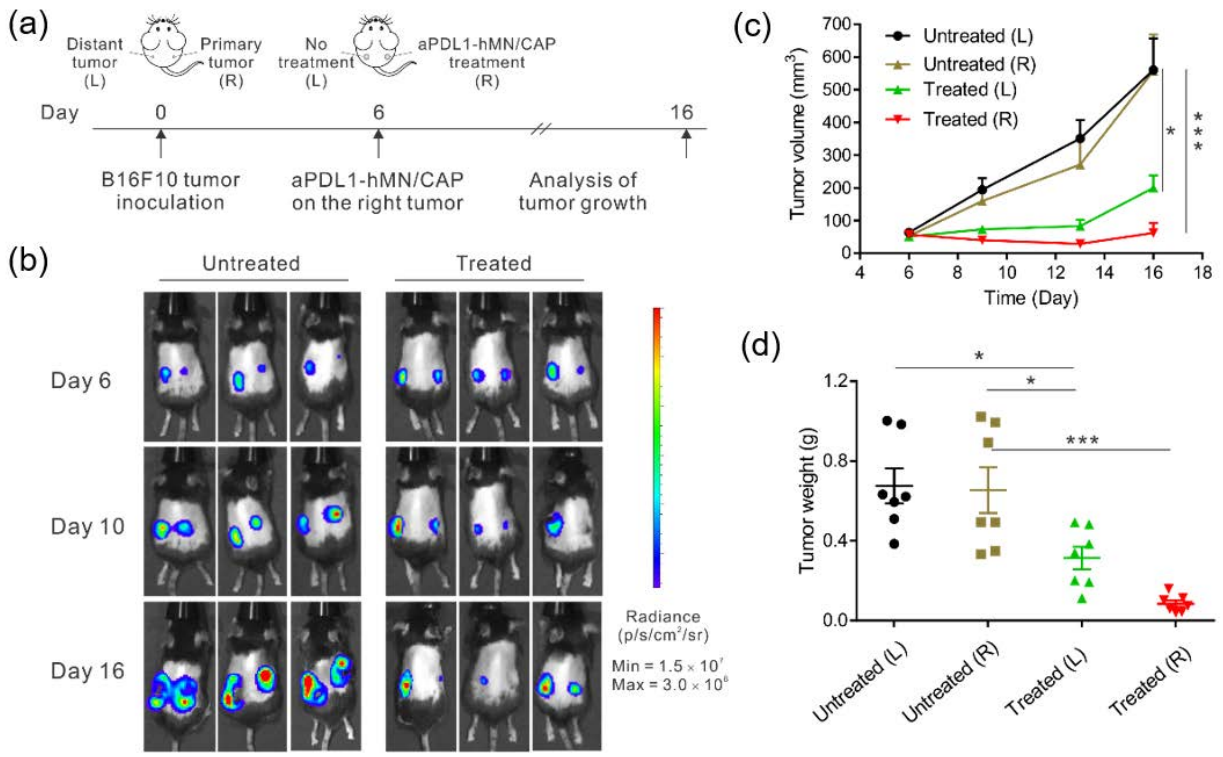

Figure 20. Plasma with immunotherapy. (a) Tumors on the right side were designated as "primary tumor" with combinational treatment, and tumors on the left side were designated as "metastatic tumor" without any treatment. (b) *In vivo* bioluminescence images of the untreated mice and treated mice treated. (c) Left and right tumor growth curves, and (d) weights in untreated and treated mice. Reproduced with permission.[361] Copyright 2020, National Academy of Sciences.

Another report by Bekeschus *et al*. also studied the effect of the composition of CAP on inducing anti-tumor immune responses.[396] It was found that the ROS composition (e.g., H_2O_2, HO•, and HOCl) in CAP changed the antitumor immunity and efficacy in a syngeneic melanoma mouse model. The ROS composition in CAP can be tuned by introducing different feeding gas compositions (e.g., Ar, Ar/O_2, He, and He/O_2). It is suggested that •OH-rich argon gas plasmas and the atomic oxygen-rich He/O_2-gas plasma exhibited the enhanced antitumor immune responses and tumoricidal effects, although authors also indicated that a more comprehensive study regarding the optimization of the feed gas composition is needed. CAP has also been investigated for cancer vaccines. Lin *et al.* used the CAP-pretreated melanoma cells as a cancer vaccine.[397] Although a small risk of tumor development (2 out of the 32 mice) in the mice received

CAP-pretreated melanoma cells, the vaccinated mice showed prolonged survival after tumor challenge.

6.3 CAP with chemotherapy

Pancreatic exocrine tumors, the most common form of pancreatic cancer, do not respond to conventional chemotherapy and radiation in most cases. Brule *et al*. evaluated a new antitumor method for topical therapy based on nonthermal plasma.[398] They developed a microplasma (plasma gun) that was used in combination with gemcitabine and found that the treatment improved tumors *in vivo* and *in vitro* more than a 36-day period, possibly due to its radiosensitization properties.

As a first-line chemotherapy agent, the alkylating agent temozolomide (TMZ) can effectively improve the overall survival rate of patients with glioblastoma (GBM). Addressing TMZ resistance and developing novel treatments are critical challenges in the treatment of GBM patients. Koritzer *et al.* proved the anticancer effectiveness of different doses of CAP in TMZ-sensitive and TMZ-resistant cells through experiments.[399] The combination of CAP and TMZ significantly inhibited cell growth and became an effective treatment for GBM. Electrochemistry therapy (ECT) has been shown to eradicate local tumors, but there are still shortcomings related to the properties of intense electric pulses (EP). Chung *et al*. developed a combination therapy of CAP and microsecond pulsed electric field (μsPEF).[400] They analyzed two different cell lines of DC-3F and B16-F10 using this method and found that the combination therapy could still improve cell membrane electropermeability and enhance the efficacy of ECT even at very low electric fields.

Zhu *et al.* combined CAP and polylactic glycolic acid as a new drug-loaded core-shell nanoparticles to encapsulate the shell of anticancer drugs.[370] The potential mechanisms of novel therapeutic approaches in targeted breast cancer therapy were also evaluated. The study demonstrated that the combination of CAP and the novel drug-loaded core-shell nanoparticles had a synergistic inhibitory effect on the growth of cancer cells compared to treatment alone. In addition, CAP induced the down-regulation of transfer-related gene expression, promoted the absorption of drug-loaded core-shell nanoparticles, and reduced the resistance to chemotherapy.

CAP affects skin barrier function, helping drugs pass through plasma-treated skin for therapeutic effects. Gelker *et al.* quantified the therapeutic effects of different plasmas by controlling voltage rise time, pulse duration, and power density.[274] The results showed that the transepithelial resistance (TEER) of all plasma-treated tissues decreased significantly, and the permeability of the hydrophilic fluorescein sodium molecule increased from 11.7 to 41.6. They found that direct treatment of excised full-thickness human skin with CAP resulted in pore formation and enhanced transdermal transport of fluorescein sodium. Rasouli *et al.* used CAP and PAM in combination with chemotherapy to selectively treat ovarian cancer cells (A2780 CP and SKOV-3 cells).[401] The results confirmed the high selectivity of PAM and the anticancer efficacy of CAP as an effective alternative to conventional treatment.

Table 1. A summary table of the examples of CAP delivery applications.

Delivery	Strategy	Applications	Performance
Direct CAP delivery	Plasma and cell/tissue interaction	Sterilization	- sterilize human tissue with minimal or no damage;[107] - damaged bacterial cell walls;[129,130] - sterilize;[152] - reduce inoculated microbial populations;[135,162,163] - kill pathogenic microorganisms, stop microbial proliferation in water[169]
		Wound healing	- speed up wound closure and promoting re-epithelialization;[183,185] - decrease inflammation *via* recruiting immune cells into the wound area and activating body-protective mechanisms;[190] - trigger fibroblasts that promote the matrix synthesis and induce the actin cytoskeleton re-arrangement;[180] - activate cytokines/growth factors related to wound healing in keratinocytes and fibroblasts;[180] - induce neovascularization[184]
		Blood coagulation	- control blood coagulation rates with minimal side effects;[194] - accelerate blood coagulation and reduce clotting times[196,202]
		Oral and dental treatment	- reduce the presence of bacteria in oral biofilms;[209] - decrease bone loss and inflammation in a rat model of periodontitis;[210] - reduce the presence of oral pathogens;[211] - increase surface adhesion;[215,218,219] - bleach or whiten tooth[228,229]
		Cancer therapy	- induce selective cancer cell death *in vitro* and tumor size reduction *in vivo*[236,237]
	Plasma-activated water	Preservation	- exhibit high antimicrobial efficacy to maintain the safety and quality of food[296]

Indirect CAP delivery		Cancer therapy	- inhibit the growth and proliferation of human cancer cells[291]
		Mouthwash	- disinfect oral pathogenic bacteria[211]
		Fertilizer	- improve seedling growth;[107] - increase germination rates[1]
	Plasma-activated saline solutions	Cancer therapy	- kill cancer cells[302]
		Disinfection	- increase the bactericidal effect[209]
	CAP-activated other media (culture medium)	Cancer therapy	- inhibit cancer cell invasion and migration, kill cancer cells[322,323]
		Disinfection	- inactivate bacteria[341]
Device-assisted plasma delivery	CAP delivery through small tube	Cancer therapy	- suppress tumor;[350] - target places or organs difficult to access for clinical applications with great flexibility[351,352]
		Anti-microbial	- target and destruct microbial with ease direct or bent access[19,117]
	CAP delivery through micro-sized tube	Cancer therapy	- kill cancer cells;[358,359] - reduce tumor size[357]
		Oral and dental treatment	- kill root canal of human tooth residual bacteria[355,356]
	CAP delivery *via* patch	Cancer therapy	- erforate the stratum corneum;[361] - translocate drug payloads into the dermis in a minimally-invasive with painless manner;[361] - overcome the limited tissue penetration;[361] - enhance anti-tumor effect compared with direct CAP[361]
Combination of CAP delivery and other therapeutics	CAP delivery with nanoparticles	Cancer therapy	- delivery therapeutic agents with CAP to solid tumors;[371] - kill cancer cells and seriously inhibit cell proliferation[370,372]
	CAP with immunotherapy	Cancer therapy	- induce immunologic memory against tumor cells[246,361]
	CAP with chemotherapy	Cancer therapy	- promote the absorption of drug-loaded core-shell nanoparticles;[400] - overcome the resistance to chemotherapy;[398,401] - induce cancer cell apoptosis[399]

7. Summary and outlook

CAP research, in particular and increasingly over the past two decades, has resulted in many promising and exciting scientific discoveries as well as the development of new technologies and applications.[402] Most notably, CAP can produce a chemically rich environment at close to atmospheric pressure and room temperature, a unique condition that can deliver highly reactive species in a beneficial and non-destructive manner. As with most technologies, progress in applications has been ahead of the elementary understanding of the associated processes and continued improvement in this process is needed to move the technology forward and to develop optimal delivery strategies. For example, CAP delivery for applications such as disinfection, wound healing, blood coagulation, cancer therapies, dental disease treatment, and others are in the early but promising stages of their development (**Table 1**). There are also multiple opportunities for collaboration and convergence with biological and biomedical sciences including, but not limited to, clinical and preventative medicine, molecular biology, and biochemistry.

CAP is establishing a new field of plasma medicine linking the interdisciplinary research works of plasma technology with biology, chemistry, physics, and medicine, even though the results obtained so far are still at a preliminary stage.[403] RONS are considered to be the key players in plasma-induced biological effects since RONS are components of regular physiological processes and, consequently, can interact with the processes of regular cell metabolism. CAP affects cells/tissues not only *via* direct delivery of itself but also by delivery of previously prepared CAP-activated medium. The delivery effectiveness of CAP-activated media increases the potential for clinical applications of plasma biomedicine because it can be prepared in advance for off-the-shelf use. CAP has been developed with different shapes and sizes for delivery purposes, especially for inside living organisms and other difficult to access regions. Further, RONS generated by CAP can be delivered for long distances which meets all treatments in human living body. However, the clinical use of biomedical devices for CAP delivery needs to be further developed and improved. With the development of images technology and plasma applications, CAP with biomedical devices could achieve precise treatment based on real-time images. Due to CAP being composed of various reactive species, it has been capable of consistently producing different

pharmaceuticals on demand and is highly adaptable. CAP is also used in combination with other drugs as both oral and injected medication.

More research is required in the future to assess the toxicity and safety of CAP delivery to humans and to clarify the mechanisms of interaction between plasma and living tissues. Although clinical application of CAP is growing rapidly, long-term and side effects need to be further evaluated through clinical studies.[17] To aid these studies, key plasma-subject interaction mechanisms must be better understood. The generation and delivery of reactive species represents a rapidly expanding and evolving multidisciplinary field of research with excitement. The multidisciplinary characteristics of CAP introduce numerous significant challenges that in turn open new opportunities. CAP research is "multi-phase" as it involves all four states of matter, for example, a propagating streamer head in the plasma state, reactive species in the gas phase, interacting solutions/media in the liquid state, and living tissue in the solid state. The mechanisms of reactive species generated in CAP and delivered to surfaces and even into the inner space of subjects are extremely complicated and require detailed studies of physical, chemical, and biological phenomena and elementary processes. Relatively limited knowledge of CAP in certain areas can pose numerous challenges and new opportunities for future research and development. It brings synergistic studies where new knowledge can be obtained by employing experimental and theoretical approaches simultaneously. Due to being very non-uniform in space and varying with time over relatively short time scales, present-day CAP diagnostics/delivery generally characterize the averaged values of the species concentrations in other words, most of the current numerical models are limited by the spatial and temporal resolution of diagnostic results. Thus, diagnostic challenges, approaches, and instruments are urgently needed for in-situ monitoring of plasma processes/delivery and accurate capture with higher spatiotemporal resolution to obtain a thorough understanding of this technology.

To date, the clinical research of plasma medicine mainly focused on the fields of dermatology, aesthetic surgery, and tumors.[403] For instance, abovementioned examples showed CAP could accelerate wound healing and promote angiogenesis; the ulcer edge on the tumor surface of 12 patients with head and neck cancer was contracted after CAP treatment. US Medical Innovations have announced results in the FDA-approved the first phase clinical trial of stage IV recurrent and

metastatic solid tumors using CAP treatment. Furthermore, one study reported that after application of nasal CAP, the mild symptoms of 3 patients with COVID-19 infection were gradually improved, with a special note that the anosmia was significantly alleviated.[404] The current studies have laid the foundation for further clinical research, but future research could advance rapidly in many medical fields. Notably, *in vivo*/*in vitro* testing of CAP treatments is needed to better understand the effects of plasma-generated ROS/RNS and other species on biomolecules, cells, biological liquids and tissues, and for the full development of plasma-based delivery therapies. Apart from the usual safety concerns, a major obstacle exists in the lack of fundamental understanding of biological, chemical, and physical mechanisms of interaction between CAP and living cells, tissues, organs, and the whole organism.

To address the abovementioned challenges, there are many ongoing projects allowing the investigation of CAP. Scientists are developing new diagnostics and numerical models to understand CAP and its delivery[98,405]. Plasma specialists are aiming to improve the utilization efficiency of CAP and reduce the waste of generated plasma. The use of solar energy as power for plasma generation in CAP technology to lower its energy consumption is also one of the hot spots[406,407]. Further and in-depth work will be needed to achieve low-cost, high-energy efficiency, suitable facilities, and convenient delivery to simplify plasma technology.

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