# Tracking the penetration of plasma reactive species into tissue models

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Abstract

Electrically-generated cold atmospheric plasma is being intensively researched for novel applications in biology and medicine. Significant attention is being given to reactive oxygen and nitrogen species (RONS), initially generated upon plasma-air interactions, and subsequently delivered to biological systems. Effects of plasma exposure are observed to millimetre depths within tissue. However, the exact nature of the initial plasma-tissue interactions remains unknown, including RONS speciation and delivery depth, or how plasma-derived RONS intervene in biological processes. Herein, we focus on current research using tissue and cell models to learn more about the plasma delivery of RONS into biological environments. We argue this research is vital in furnishing an underpinning knowledge required to realise the full potential of plasma in biology and medicine.

Keywords: Cold atmospheric plasma, tissue model, reactive oxygen and nitrogen species, cell membrane, cancer

**Why the interest in cold atmospheric plasma in biological and medical sciences?**

Over the past two decades interest in the application of **cold atmospheric plasma** (herein referred to as plasma) in biology and medical sciences has been rapidly growing. There is significant optimism that plasma can be developed for a wide range of medical applications such as for the treatment of chronic wounds [1], cancers [2], dental decays [3] and dermatological indications [4]. In biotechnology, plasma is being investigated for enhancing **cell transfection** efficiency [5-7], **stem cell differentiation** [8] and **tissue regeneration** [9]. A number of plasma sources are being commercialized for medical use [10, 11]; these sources are being extensively characterized and optimized to ensure they can be applied safely to tissue [4, 10]. Significant progress in the applications of plasma has opened a new research field termed **plasma medicine**; with the core being the use of cold atmospheric plasma (as opposed to thermal plasma) to intervene in biological processes for improving medical outcomes.

In parallel, plasma is being applied to animal and plant tissues to enhance the quality and value of foods (meats, fruits and vegetables) [12], improve seed germination and plant growth [13, 14] and treat plant disease [15]. Plasma is attractive as it is cost effective, does not necessarily change the edible qualities of food, increases food shelf-life and can be applied to packaged food [16, 17]. Moreover, plasma addresses food safety problems, such as the reduction of salmonella infections on egg shells in Europe [18].

Although plasma research (applied to tissues and cells) has advanced in Europe, South East Asia and USA, we argue that there is an urgent need almost to 'double-back' and with a renewed focus on the fundamentals of how plasma interacts with tissues. This is a topic that we consider has been largely been overlooked worldwide: yet is crucial in both the context of health and disease in human tissue [19] and in the deactivation of microbes on food products [20, 21].

***The purpose of this Opinion Article is to put a unique focus on the speciation and transport of plasma-generated RONS in tissue and cells. We will argue***

***that such a focus will furnish vital underpinning knowledge that will help put plasma technologies on a firmer scientific footing.***

**Plasma as a source of RONS**

Our starting point is that when plasma reacts with the ambient air, it generates a rich mixture of reactive oxygen species (**ROS**) and reactive nitrogen species (**RNS**), or collectively **RONS**, which are most likely to play the major role in the phenomenon described to date [22]. Although we readily acknowledge possible contributions from other plasma constituents, such as photons, metastables, charged species and strong electric fields [19, 23], these are outside the scope of this opinion article.

In the context of human health, historically, RONS have been thought to be 'bad' and associated with **free-radical ageing**; but current thought now focuses on the role of RONS in a myriad of biological processes that are implicated in the protection and repair of cells and/or organisms [24]. And, whilst large doses of RONS to any cell/organism are no doubt 'harmful', the delivery of small doses of specific RONS could be beneficial in the treatment of a wide range of indications [22, 24]. For example, wound healing can be enhanced by small doses of exogenous $\mathbf{H_2O_2}$, but higher doses delay wound healing [25]. The efficacy of plasma (when applied to tissue) is almost always linked to RONS that are naturally produced *in vivo*. And the argument follows that RONS regulate key biochemical pathways within intra- and intercellular environments, inducing chemical and physical changes in cells [22, 24].

***But, the intervention of plasma-generated RONS in cellular processes assumes a priori the availability of RONS.*** This is quite an assumption considering the relatively short lifetime and diffusion distances and high reactivity of many plasma-generated RONS; e.g., the $\mathbf{OH^{\bullet}}$ is calculated to have a lifetime of $10^{-9}$ seconds with a diffusion distance of 0.009 micrometres within mammalian cells [26]. However, even before the plasma-generated RONS enter cells, the RONS need to traverse at least three major barriers at the (1) plasma-fluid, (2) tissue fluid-tissue and (3) tissue-cell (Figure 1) interfaces, before finally reaching the cell interior. During their passage through the physiological environment, RONS will face additional obstacles such as extracellular matrix (ECM) proteins and antioxidants.

Therefore, it is remarkable how plasma can essentially non-invasively destroy cells deep within **biofilms** [27] or cancerous **tumours** [28] when these cells may be buried tens of micrometres to even millimetres away from surfaces directly accessible to plasma.

Computational modelling has been utilised to provide further insight into the plasma delivery of RONS into tissue fluid and tissue [29, 30]. These simulations determined that plasma can deliver **$O_3$**, $H_2O_2$ and **$NO_x$** to tissue covered with a thin liquid layer; but the presence of alkane-like hydrocarbons in physiological fluid can inhibit the delivery of ROS [29]. In biofilms, $H_2O_2$ and **$O_2$** can potentially quite deep to millimetre depths, whereas the penetration depth of other ROS such as $O_3$ was limited to 5-40 micrometres [30].

To detect the plasma-generated RONS in aqueous solution, **ESR** [31, 32], **LC-MS** [33, 34] and **UV-Vis** [35-37] are often used. Usually ESR is used to measure short-lived RONS in aqueous solutions treated by plasma (including the $OH^{\bullet}$ with a lifetime of < 100 microseconds, **ONOOH** with lifetime of ~1 millisecond, and **$O_2^-$** and **HOO•** with lifetimes < 10 seconds). However, the consensus is that the shorter-lived RONS rapidly decay in aqueous solution to give more stable longer-lived secondary RONS such as **$NO_2^-$**, **$NO_3^-$** and $H_2O_2$; these RONS have been readily detected by LC-MS, FT-IR, and UV-Vis analysis of plasma treated aqueous solutions.

**Comment [MP1]:** Two comments here: first, "lifetime" is a bit ambiguous. Do you mean "half-life"? Second, can you include citations for these numbers?

Aggregated, these data lead to a number of obvious questions:

- What RONS, originating from plasma (or RONS produced downstream), are delivered into real biological targets (animal, food product, plant) and how far are they delivered?
- How do these RONS interact with the various components of a real tissue, e.g. in an animal, tissue fluid, extracellular matrix, cell membranes and intracellular components.

Herein, we describe the progress made to date in developing models to measure the sub-surface (deeper than the top few nanometers to millimeters) interactions of RONS in tissues, and in the later section in cells. In order to reach the surface of

and/or penetrate a target cell interior, we argue that three major interfaces must be breached (shown in Figure 1). In the case of more complex 'targets', such as cancer with an associated wound, there may be clotted blood, pus and wound debris (Figure 1).

Experimental and simulation studies clearly show that deeper within tissue fluid and tissue (millimetres below the surface) most of the RONS from the plasma-air interface are converted to more stable RONS with a longer half-time – usually $H_2O_2$, $NO_2^-$, $NO_3^-$, as well as $O_2$ [35, 36, 38]. These RONS are produced in the liquid phase from chemical reactions with neighbouring RONS and also with the constituents of the tissue fluid and tissue. Plasma treatment can also stimulate the intracellular production of RONS, which may amplify the original RONS dosage delivered by plasma into tissue fluid and tissue [39, 40].

**Delivery of RONS into tissue – the gap in our knowledge**

Leading experts in plasma biotechnology have argued that plasma stimulates tissue sub-surface by **cell-to-cell communication** – whereby only surface cells are directly exposed to the plasma (or effluent) and any subsurface effects are transmitted from these cells [41-43]. This explanation fits with biological theory. But it still cannot explain how the RONS reach the surface layer of cells in the first place. What is perhaps a little underappreciated is that in open wounds (Figure 1) there are major physical barriers to traverse, comprising congealed blood and pus from infections. For further information, refer to figures 1-4 in reference [44].

In this opinion article, we are concerned with the transport of more stable RONS into tissue and these RONS interacting with the first 'physical barrier' of the cell (i.e. the phospholipid cell membrane). This is not to minimise the importance of, for example, RONS-DNA interactions – which could have significant implications for the safety of plasma treatments (e.g. chromosomal stability and genotoxicity [45, 46]) – but are outside of the scope of this article.

**Delivery of RONS into tissue models**

One first step toward modelling RONS penetration and delivery into tissue was a simple experiment using a **plasma jet** and a gelatin target with embedded

phospholipid vesicles [47]. The vesicles encapsulated a self-quenched dye that when released (upon vesicle destruction) resulted in a clear fluorescence switch-on. These very first data showed that a plasma jet could interact (disrupt) phospholipid cell membranes at least several hundred micrometres below soft hydrated material. A second follow-up study showed that the spatial surface distribution and penetration depth of plasma-generated RONS was very similar to the patterns observed for vesicle destruction [48]; and prolonged treatment enabled ROS to be delivered to millimetre depths in the gelatin target by the plasma jet. Apart from two initial studies by Fridman and colleagues using agarose tissue models [49, 50], there do not appear to have been any earlier similar studies on tissue penetration.

This was surprising because in the development of any new medical therapy, usual questions asked at some point (and often asked by the regulator) concern:

- What are the active species?
- What is the mode of action?
- What are the safe doses?

These questions need to be addressed in the development of any new medical therapies. In plasma medicine, knowledge of the plasma-phase species is important, but knowledge of those delivered into any biological system is vital.

A subsequent development to the approach described above used gelatin and agarose model targets with either embedded chemical reporters, or simply as films through which plasma-generated RONS traverse, to measure RONS speciation and delivery into and through tissues [36, 37, 48, 51-54]. In the latter case, UV-Vis spectroscopy was used to identify RONS that traverse the targets. Gelatin was chosen because it consists of collagen, which is a major protein in skin, bone and connective tissue [55]; gelatin also provides a barrier to $H_2O_2$ (when spotted as solution on top of the gelatin surface) and potentially to other RONS [48]. Agarose was chosen because it has been widely utilised to mimic different tissue types (e.g. skin, liver, brain) [56, 57] and has been utilised as a tissue model for studies in

radiotherapy and neuroscience [58, 59]. The physicochemical properties of gelatin and agarose can easily be tailored for specific applications.

Experiments with the tissue models have revealed the key RONS delivered into tissue (speciation) and likely relative (but not absolute) concentrations (doses) and depths of delivery. From these findings it is clear that plasma treatment can induce physical and chemical modifications deep within tissue to millimetre depths. Since our earlier studies, other groups have started utilising gelatin and agarose tissue models with different plasma sources and also observed relatively deep penetration of RONS to millimetre depths [60, 61].

However, whilst they are useful for gaining new insights into plasma-tissue interactions, these models are relatively poor mimics of real animal tissues, lacking proteins, enzymes, antioxidants, cells, or a heterogeneous structure (e.g. skin layers: epidermis, dermis, hypodermis). And current tissue models do not mimic the gelatinous-like structure of biofilms. Building in these 'features' of real tissues would provide much more robust models to explore the plasma delivery of RONS into tissue. Very realistic 3D models of mammary glands [62] and the extracellular matrix [63, 64] have been developed in recent years; future plasma medicine research should adopt these models.

Concomitant with RONS delivery, plasma jets were also shown to oxygenate the tissue models [36, 52, 54]. Tissue oxygenation with a plasma jet has also been observed *in vivo* by Collet and colleagues, who have shown that a plasma jet induces tissue oxygenation in live mice [65]. This result suggests that plasma jets can counteract hypoxia, a phenomenon that impedes healing in chronic wounds and increases resistance of cancer cells to radiation and cytotoxic drugs [66].

However, as noted above, we expect that gelatin would present a barrier to $H_2O_2$ [48], and by inference to other RONS. Therefore, an important question arises: what is the 'force' that is responsible for the plasma delivery of RONS across what should be an impenetrable barrier? We argue electric fields from the plasma jet have an important role. One study observed that plasma jets deliver RONS onto the surface of gelatin tissue models in a star-shaped pattern [48]. Similar star-shaped patterns

were observed for plasma surface streamers across insulators as measured by **Pockels sensing** and seen in **Litchenberg figures** [67]. Recently, it was shown that a relatively small electric field of 20 V $cm^{-1}$ can significantly enhance the plasma delivery of RONS into a gelatin tissue model [60]. These results indicate that electric fields facilitate the plasma delivery of RONS into real biological tissue. However, the data is still relatively sparse, so the results are obviously far from conclusive and much further work is needed to understand mechanisms that drive plasma-generated RONS deep into tissues.

**Crossing the cell membrane barrier**

The phospholipid membrane provides a barrier to exogenous RONS. Understanding plasma-generated RONS interactions with cell membranes is vital to developing a fuller mechanistic picture of how plasma potentially stimulates cells. Broadly, one of two events can occur when RONS encounter the cell membrane: (i) RONS can react with the cell membrane, or (ii) RONS cross the cell membrane (Figure 2). With renewed interest and research dedicated towards elucidating the molecular mechanisms of lipids in their contribution to diseases, there is growing optimism that new and more effective approaches can be obtained to prevent and cure diseases such as cancers [68].

Computer simulation experiments have improved our understanding of plasma interactions with cell membranes. For example, molecular dynamic simulations have shown that reactions of RONS with the cell membrane lead to lipid peroxidation, enhancing the ingress of further RONS (Figure 2) [69]. Electric fields from plasma can also act in synergy with plasma-generated RONS increasing membrane permeability [70]. Cholesterol in healthy cell membranes can significantly reduce the ingress of RONS, which is particularly important for targeted cancer therapy because cancer cells generally have a lower percentage of cholesterol in their cell membrane [69].

Computer simulations have been supported by experiments with models of cell membranes [71-74]. Other results showed that plasma can directly deliver RONS across phospholipid membranes in simple buffers and in proteinaceous solutions required for *in vitro* cell culture [75-77]. These studies tailored the vesicle diameter to

mimic eukaryotic and prokaryotic cells and observed that due to the large differences in the surface area:volume ratio, plasma delivers a higher dosage of RONS into prokaryotic cells, which is important for decontamination of wounds infected with bacteria [75].

Whilst these approaches have yielded valuable insights into how lipids and cholesterol in cell membranes react with and influence the ingress of RONS into the cell interior, there are a number of clear limitations in current simulations. These include:

- Real cell membranes are made up of many different lipids (rather than one or two typically used in computational and experimental models). In fact, eukaryotic cells synthesise thousands of different lipids [78].
- Different healthy cell types have membranes that differ significantly in composition. The variability in cell membranes is further complicated in diseased cells, where membrane composition can change with disease status, e.g. in cancer cells [68].
- Eukaryotic cells possess internal membranes that encase their nucleus and organelles with each membrane being different in composition and function. This further increases the complexity of the cell membrane properties that present a barrier to RONS (i.e. nuclear and organelle membranes).
- Cell membranes are dynamic and can self-repair.
- Real cell membranes contain proteins and are decorated by complex sugars.
- Cell membranes contain channels that actively move molecules in and out (Figure 2), such as **aquaporins** [79].

Some of this complexity could be readily incorporated into synthetic cell membranes, such as more complex lipid compositions. Other features, e.g. the decoration by complex sugars, would require considerably more thought about how to achieve. Further aspects, such as ion channels, may not be technologically achievable at this point in time and we would consider, at least for present, it would perhaps be better to progress to real cells.

**Are plasma-generated RONS really intervening in biological processes?**

One significant challenge we have already highlighted is to demonstrate that it is indeed the plasma-generated RONS (or its progeny) that ultimately 'intervenes' in a biological pathway, whether intra- or intercellular, to bring about some upstream medical outcome. In this respect, more sophisticated tissue models (e.g. with embedded cells) and models of cell membranes could be very powerful. Adapting the method of Gorbanev and colleagues [80], using isotopically labelled reagents in either the gas feed, or ambient atmosphere, it should be possible to determine both the origin and final fate of specific RONS, from the plasma into individual cells.

Eventually, pre-clinical models will be required to validate any results generated with the tissue/cell models. But we argue that tissue/cell models will always remain important tools in plasma medicine research because they enable more time and cost effective experimentation and reduce the number of animal experiments, which would also eliminate unnecessary animal suffering.

**Concluding remarks and future directions**

A better understanding of plasma-tissue/cell interactions will enable us to develop plasma applications that are safer, more robust and effective. Simple models of tissue and cells will aid in developing this understanding. Future research with these models could address some important questions (see Outstanding Questions). In answering these questions, the potential immediate impacts include:

1. Plasma medicine and health – the long term goal (and major impact) is the use of plasma to synthetically generate RONS that intervene in known biological processes associated with disease or tissue regeneration.
2. Food manufacturing – outside of medicine, a likely impact would be the use of RONS to enhance food manufacturing (meats, fruits and vegetables).

In summary, developing synthetic models to mimic the chemical, physical and biological architecture of biological tissue remains a great challenge. But tissue models do offer advantages compared to experimentation with cells and animals including simpler, faster and more cost-effective experimentation, and avoid growing ethical concerns associated with animal experimentation. Incorporation of living cells into the tissue models is a logical next step to understand the role of cell-to-cell signalling in the plasma treatment of diseased tissue. Perhaps even more pertinent,

is the use of tissue models to unravel the role of electric fields in the delivery of RONS into tissue. Understanding how to effectively harness electric fields in plasma medicine should facilitate the development of more effective plasma medical technologies.

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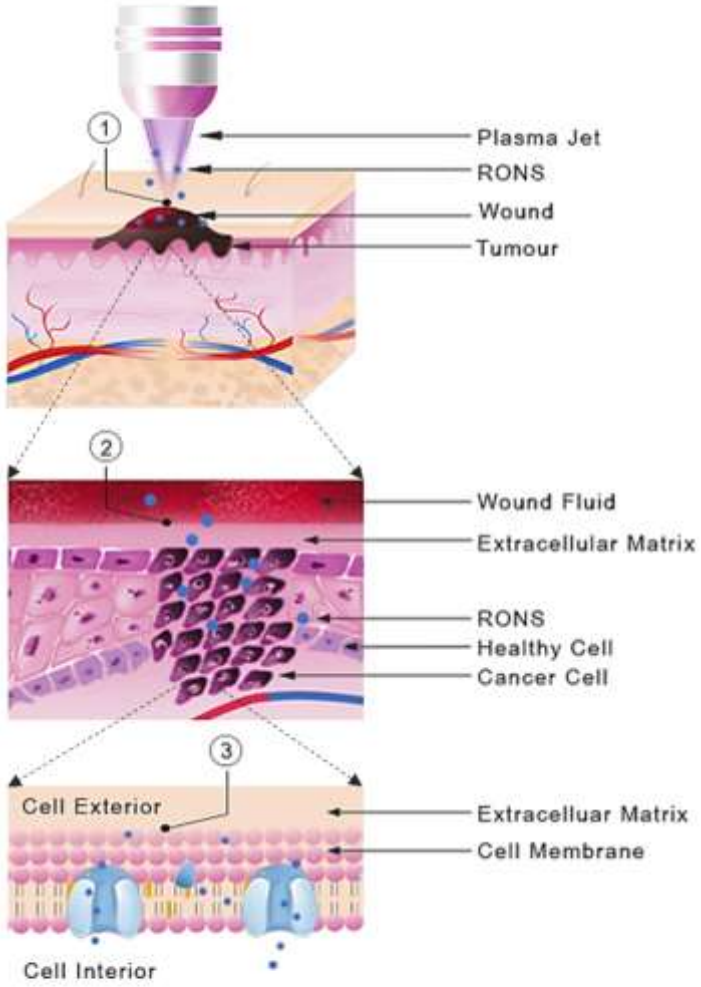


**Figure 1 (Key Figure).** Cold atmospheric plasma jet delivery of RONS into a cancerous tumour with an associated wound. The plasma-generated RONS need to traverse at least three major interfaces before reaching the interior of a cancer cells (from top to bottom): (1) plasma-fluid, (2) tissue fluid-tissue and (3) tissue-cell. Cancerous tumours are often associated with ulcerous wounds consisting of congealed blood and pus that present an additional obstacle to RONS.

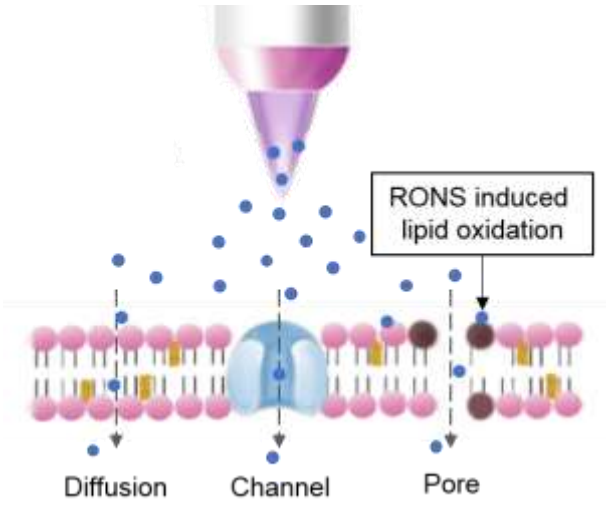


**Figure 2.** Mechanisms by which plasma-generated RONS can cross a cell membrane barrier to reach the cell interior. In order from left to right: through a passive diffusion process, or through a membrane channel, or through pores generated by lipid oxidation. Strong electric fields (not shown on the illustration) from the plasma jet may further facilitate RONS ingress by inducing cell membrane pore formation.

**TRENDS BOX**

- Physical effects of plasma can be seen to depths of several hundred micrometres within tissue.
- Plasma-derived RONS are likely to be delivered millimetres into tissues.
- Speciation reveals that RONS delivered by plasma into tissue fluid and tissue are predominately stable secondary RONS – e.g. $H_2O_2$, $NO_2^-$ and $NO_3^-$.
- The plasma generation of RONS within a hydrated target is influenced by the target matrix that can enhance or reduce the RONS concentrations and act as a reservoir RONS.
- It is likely that the concentration of these plasma-derived RONS exceeds hundreds of micromoles, even at depths of several millimetres within tissue.
- Oxygen concentration at the time of plasma treatment significantly influences RONS generation within a hydrated proteinaceous target.

**OUTSTANDING QUESTIONS BOX**

- What are the kinetics and mechanisms in the transport of plasma-derived RONS into tissues and cells? And what proportion of these RONS originate from the plasma-phase *versus* secondary RONS?
- Can we tailor plasma to deliver specific RONS to targeted depths and specific sites within tissue?
- How does the biological target influence the speciation and depth in the plasma delivery of RONS? Do biological targets 'quench' RONS, or provide a reservoir/further source of secondary RONS?
- Can we eventually describe plasma treatment in terms of a RONS dose (similar to a medical drug)?
- Does plasma elevate intracellular RONS directly through the delivery/generation of RONS into cells, or indirectly through the modification of the extracellular matrix?
- What is/are the responsible force(s) for the transport of primary or secondary plasma-derived RONS in tissue? Can we manipulate these to obtain more effective therapies?
- How does the composition of the cell membrane influence the ingress of plasma-derived RONS into the cell interior? How important is the cell membrane composition compared to cell ion channels?
- Can plasma-derived RONS cross internal cell membrane barriers?
- Does the membrane surface area:cell volume ratio influence RONS ingress? This is particularly important for plasma decontamination of wounds infected with small organisms (e.g. bacteria).

## GLOSSARY

| | |
|---|---|
| Aquaporins | Channels found on the cell membrane that help facilitate the passage of $H_2O_2$ into and out of the cell |
| Cell transfection | |
| Cold atmospheric plasma | Electrical plasma in the form of a partially ionized gas operated at atmospheric pressure without considerably heating the background gas above ambient temperature |
| Biofilms | Groups of adherent microorganisms (e.g. bacteria cells) forming colonies surrounded by a polymeric matrix |
| Cell-to-cell communication | |
| ESR | Electron spin resonance |
| Free-radical ageing | |
| $H_2O_2$ | Hydrogen peroxide |
| HOO• | Hydroperoxyl radical |
| LC-MS | Liquid chromatography mass spectrometry |
| Litchenberg figures | |
| $NO_2^-$ | Nitrite |
| $NO_3^-$ | Nitrate |
| $NO_x$ | Nitrogen oxides |
| $O_2$ | Molecular oxygen |
| $O_2^-$ | Superoxide |
| $O_3$ | Ozone |
| $OH^•$ | Hydroxyl radical |
| ONOOH | Peroxynitrous acid |
| Plasma jet | Cold atmospheric plasma typically formed in a dielectric tube with a flowing inert gas (e.g. helium or argon). The tube is open-ended enabling the plasma to extend into the ambient atmospheric giving a 'jet-like' appearance. |
| Plasma medicine | Use of cold atmospheric plasma to induce a physical |

| | |
|---|---|
| | or chemical change in tissue fluid, tissue or cells to induce a desired biological response or treat a disease. |
| Pockels sensing | |
| RNS | Reactive nitrogen species |
| RONS | Reactive oxygen and nitrogen species |
| ROS | Reactive oxygen species |
| Stem cell differentiation | |
| Tissue regeneration | |
| Tumours | Abnormal masses of tissue growth, which can be benign or cancerous |
| UV-Vis | Ultra-violet visible spectroscopy |