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Modelling the helium plasma jet delivery of reactive species into a 3D cancer tumour

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Abstract

Cold atmospheric plasmas have attracted significant worldwide attention for their potential beneficial effects in cancer therapy. In order to further improve the effectiveness of plasma in cancer therapy, it is important to understand the generation and transport of plasma reactive species into tissue fluids, tissues and cells, and moreover the rates and depths of delivery, particularly across physical barriers such as skin. In this study, helium (He) plasma jet treatment of a 3D cancer tumour, grown on the back of a live mouse, induced apoptosis within the tumour to a depth of 2.8 mm. The He plasma jet was shown to deliver reactive oxygen species through the unbroken skin barrier before penetrating through the entire depth of the tumour. The depth and rate of transport of He plasma jet generated H_2O_2 , NO_3^- and NO_2^- , as well as aqueous oxygen [$O_2(aq)$], was then tracked in an agarose tissue model. This provided an approximation of the H_2O_2 , NO_3^- , NO_2^- and $O_2(aq)$ concentrations that might have been generated during the He plasma jet treatment of the 3D tumour. It is proposed that the He plasma jet can induce apoptosis within a tumour by the 'deep' delivery of H_2O_2 , NO_3^- and NO_2^- coupled with $O_2(aq)$; the latter raising oxygen tension in hypoxic tissue.

Supplementary material for this article is available online

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Keywords: tissue oxygenation, tissue model, plasma jet, hypoxia, hyperbaric medicine, reactive oxygen species (ROS) and reactive nitrogen species (RNS), cancer therapy

1. Introduction

In the context of human health, historically, reactive oxygen species (ROS) and reactive nitrogen species (RNS), or collectively RONS, have been thought to be 'bad' and have long been associated with free radical ageing [1]. However, current revised thought now focuses on the role of RONS in a myriad of normal intercellular and intracellular biological processes [2]. RONS are implicated in the protection and repair of cells and/or organisms [2, 3] where RONS regulate key biochemical pathways, inducing chemical and physical changes within cells and signalling between cells, particularly in the context of disease [4–8]. Consequently, it is now considered that the controlled delivery of small, monitored doses of specific RONS could be beneficial in the treatment of a wide range of medical indications including cancers and infected wounds [2, 8–11].

Despite the potential benefits, effective and safe methods for the direct delivery of RONS to selected cell populations within tissue have yet to be established. Of the challenges in the administration of RONS there is first, the short half-life and limited diffusion distances of many RONS (a consequence of their high level of reactivity), and when applied topically (e.g. to skin) this may prevent RONS from penetrating deeper into tissue [12]. Secondly, it is difficult to deliver RONS at an optimal dose and rate to diseased cells within a tissue mass without inducing other unwanted effects in healthier, or normal cells as a consequence of the potential cytotoxic and genotoxic effects of RONS [13, 14]. In the treatment of cancer tumours, ionizing radiation in the form of x-rays or γ -rays penetrate tissue creating a rich source of ROS, which are implicated in the destruction of cancer cells [15]. Although ionizing radiation is a highly effective and widely accepted form of cancer therapy, it is highly damaging and radiation associated skin injury is likely to occur in 95% of patients who receive radiation therapy [16].

An additional challenge, worth noting in light of the data presented in this study, is in the destruction of tumours, where there is the potential issue of cancer cells residing within hypoxic tissue. The concept of hypoxic regions within tumours dates back to 1909 and it has long been suspected that under low oxygen tension, cells are 'afforded' radiation protection [17]. The idea of raising the concentration of oxygen in tissues at the time of radiotherapy was the subject of a seminal paper by Gray et al [18]. A meta-analysis of 83 randomised trials showed a clear benefit from raising tissue oxygen tension during radiation therapy, with the clearest benefits seen in cancers of neck and head [18]. However, there are only a limited number of ways to raise oxygen tension in tissues, which include breathing pure oxygen at or above standard temperature and pressure, or hypoxic modification by the means of an administered drug (e.g. nimorazole) [17, 19, 20]. Likewise, raising oxygen tension in tissue to promote healing in chronic wounds, is the basis of hyperbaric medicine [21].

Cold atmospheric plasmas, herein referred to as plasmas, provide an alternative, non-ionizing source of potentially highly reactive and excited state species. Over the past two decades, significant optimism has developed for the potential of plasma in healthcare [22–25]. Externally applied plasma can be used to both eradicate cells or stimulate cellular activity without skin injury, depending upon dose [26]. Plasma is being investigated for the treatment of chronic wounds [23, 27–34], bacterial infections [34–36] dental hygiene [37] and decay [38, 39] and as a new and/or adjunct technology in the treatment of cancers [40–53].

These plasmas impinging on, or mixed with air, provide a rich source of RONS including nitric oxide (NO), atomic oxygen (O), hydroxyl radical (OH[•]), peroxynitrite (ONOO⁻) and hydrogen peroxide (H₂O₂) [24, 54]. In most studies, the efficacious effects of plasmas (when applied to tissue) are nearly always linked to RONS that are produced in vivo [55]. Hypotheses have been put forward to explain how plasmagenerated RONS intervene in cellular signalling processes to destroy cancer cells whilst not damaging the surrounding healthy cells within the tissue [46–48, 56]. These hypotheses assume a priori the availability of RONS. However, the mechanisms by which externally, plasma-generated RONS first access and then, for example, eradicate cells within tumours [57, 58] (which could be millimetres in thickness), requires further consideration. Based on a well-established literature on the interactions of reactive species generated by plasmas with 'solid' organic surfaces it would be expected that the effects from plasma are limited to the uppermost surface regions (1–2 nm) of organic targets [59–62]. Even in 'softer', hydrated biological matter it should be anticipated that the RONS arriving at a surface react on a very fast timescale, and thus have limited penetration [8, 12]. This has led key opinion leaders to propose that the deeper tissue effects of plasma treatment arise from cell-cell communication events [12, 46, 63].

Given the importance ascribed to plasma-generated RONS in cancer therapy, the identification and quantification of RONS that are delivered within plasma-irradiated tissues remain largely overlooked [64]. Previous research has shown that helium (He) and argon (Ar) plasma jets efficiently deliver RONS to millimetre depths into gelatin [65–67] and agarose [68–73] tissue models, and across phospholipid membranes of cell models [74–76]. A recent study has shown that a plasma jet delivers RONS through real biological tissue [77], which supports the results in the tissue model experiments.

In this study, the effect of He plasma jet treatment on cancer cell apoptosis within an approximately 3 mm thick tumour, grown on the back of a live rodent, was first analysed. These results were correlated to the depth of ROS delivered by the He plasma jet directly into the tumour. Then, an agarose tissue model of varying thickness was used to mimic the barrier to that encountered when the plasma jet contacted the mouse skin. Agarose was ideal for this study because it has been previously used as a tumour-mimic for training and studying radiofrequency ablation [78-80] and as a tissue model in radiotherapy studies [81]. The agarose tissue model was used to determine the speciation, depth and rate of RONS and aqueous oxygen $[O_2(aq)]$ delivery from the He plasma jet. The major longer-lived plasma generated molecules were measured: H₂O₂, implicated in a range of cellular processes [82–85], and nitrate (NO_3^-) and nitrite (NO_2^-) , which are now the focus of much recent research as alternative sources (to arginine) for nitric oxide (NO) production [86]. $O_2(aq)$ was measured because plasma jet treatment can also potentially raise the oxygen tension within a hypoxic cancer tumour. Overall, the results in this study are used to explain the potential mechanisms, with respect to the plasma delivery of reactive species into tissue, of how He plasma jet treatment can reduce the size of cancer tumour growth without significantly damaging the skin.

2. Experimental

The He plasma jet used in this study is described in detail elsewhere [70, 71, 87]. Briefly, it consisted of a 150 mm long glass tube, tapered from an inner diameter of 4 mm to 800 μ m at the nozzle. He gas was fed into the glass tube with a fixed gas flow rate of 1 standard litres per minute (slpm). The He plasma jet has a 15 mm long copper external ring electrode wound onto the glass tube at a distance of 40 mm from the nozzle. A high voltage bipolar square wave pulse of 7 kV (peak-to-peak) at 10 kHz was applied to the external electrode. The glass tube and powered electrode were placed in a 10 mm thick PTFE housing to shield the high voltage electrode for safety. The length of the plasma plume was 12 mm with the operating conditions described above. The same He plasma jet parameter was used for all the experiments in this study. He plasma jet delivery of RONS and $O_2(aq)$ directly into deionised (DI) water and through an agarose tissue model were measured in real-time with a conventional double-beam ultraviolet-visible (UV-vis) (spectrophotometer (U-3900, Hitachi), which was used to measure the UV absorbance of DI water inside a quartz cuvette (100-QS, Hellma Analytics) with a standard optical path of 10 mm. All agarose tissue models contained 2% (w/v) agarose and 98% DI water.

The transport of ROS through pig skin was monitored using a chemical ROS indicator, 2,7-Dichlorodihydrofluorescein diacetate (DCFH-DA). The pig skin was obtained as a waste product from a pig that was sacrificed for food. DCFH-DA (Sigma-Aldrich, catalogue number D6883) was prepared using a similar protocol to the one described by Low *et al* [88]. A 2 mM stock of DCFH-DA was prepared in ethanol and stored at -20 °C until use. Ester hydrolysis was induced by adding 500 μ l of stock DCFH-DA to 2 ml of 10 mM NaOH and incubating for 30 min at 25 °C in the dark. The solution was then neutralised by adding 10 ml of phosphate buffered saline (PBS) (Sigma-Aldrich, catalogue number P4417), pH 7.4. The solution was stored at 4 °C in the dark until use. Fluorescence of oxidized DCFH was recorded using a BMG Labtech Fluostar Omega microplate reader. Three measurements of 100 μ l aliquots of the test solutions were recorded for each experiment in a 96-well microplate (Costar); experiments were performed in triplicate (total n = 9measurements per treatment group). Fluorescence measurements were recorded at excitation and emission wavelengths of 485 nm and 520 nm, respectively, with a gain setting of 900. The normalised fluorescent intensity was calculated according to the following formula: (DCFH indirectly treated with the He plasma jet or He gas flow or H₂O₂ solution spotted on pig skin/untreated DCFH)–1.

For the rodent tumour model study, the seven week old male BALB/c nu/nu mice used in the study were injected in the dorsal region subcutaneously with 4×10^{6} 253JB-V cells in 100 μ l of Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. The highly metastatic human bladder carcinoma cell line 253JB-V, is a sub-line derived through orthotropic cycling of the non-tumourigenic human UC cell line 253J-P. The mice were housed in plastic cages with stainless steel grid tops in an air-conditioned room with a 12 h light-dark cycle maintained at 25 °C and fed with water and food ad libitum. The animal experiments were conducted in accordance with the institutional guidelines and regulations reviewed by the animal experiment and welfare committee of Kochi Medical School. The tumours were monitored until they reached a thickness of 2.8 ± 0.5 mm. Each mouse was exposed to the He plasma jet or He gas flow for 15 min. After treatment, the mice immediately received intraperitoneal injection of 500 μ l Luminol solution prepared in sterile saline. luminescence imaging was carried out 10 min after treatment (Xenogen IVIS Spectrum 200). Following 24 h after the He plasma jet or gas flow treatment, all mice were sacrificed and the tumours were removed and examined. In pathological examinations, the resected tumour sections were stained using the terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) method and with hematoxylin and eosin (H + E). Harvested tumours were fixed in 20% formalin at room temperature for 48 h. Each tumour was processed into paraffin and cut into 10 μ m thick sections. The sections were then stained with H + E using standard protocols. For the TUNEL assay, the sections of paraffinembedded specimens were de-paraffinized and treated with 1:500 proteinase K solution $(20 \,\mu \text{g ml}^{-1})$ for 15 min. The sections were incubated with terminal-deoxynucleotidaltransferase buffer, followed by incubation with peroxidaseconjugated streptavidin (1:400) and diaminobenzidine and staining with Gill's hematoxylin.

Statistical analysis was performed on the data presented in figures 2 and 3, using an unpaired Student t-test assuming unequal variances. A p value of less than 0.05 or 0.001 was considered to be significantly different (95% and 99.9% confidence, respectively).

Details of how the discharge voltage and current waveforms and optical emission of the plasma jet were measured, the UV–vis spectroscopy method, and the method used to prepare the agarose tissue model are provided in the



Figure 1. Photograph showing the He plasma jet treating the cancer tumour on the back of the mouse.

supporting information, S1, which is available online at stacks.iop.org/PSST/27/014001/mmedia.

3. Results

Tumours were treated with a He plasma jet as shown in figure 1, or with the neutral He gas flow without plasma (i.e. with no applied voltage). Twenty four hours after treatment with the He plasma jet or gas flow, the rodents were sacrificed and the induction of apoptosis within the tumour was examined by taking histological sections to a depth of 2.8 mm within the tumour. The cells were stained for DNA fragmentation, a characteristic hallmark of apoptosis, using the TUNEL method, and counter-stained with H + E to visualise all DNA and protein within the tissue section. Figures 2(A)and (B) show full sections of resected tumours stained by H + E or by TUNEL. In these, the top of the tumour that was in the direct line-of-sight of the (A) neutral He gas flow or (B) He plasma jet are marked with arrows. The H + E staining reveals no gross changes in the tumour apart from a slight depression at the point of the plasma jet contacting the skin surface, which was also observed on the agarose tissue models (supporting information, S2). Figure 2(C) is an expansion of a TUNEL stained region of the tumour following He plasma jet treatment to show the difference in colour for cells stained positively with TUNEL (marked with arrows) compared to unstained cells. These marked cells were scored as a function of depth into the tumour (figure 2(D)) and the average counts were plotted for the He gas flow and He plasma jet treated tumours in figure 2(E). From figure 2(E), it can be clearly seen that the number of TUNELstained positive cells were significantly greater in the plasma jet treated tumour, when compared to control, i.e. He gas flow (n = 5, p < 0.001).

One possible explanation for the induction of apoptosis within the tumour could be that the plasma jet stimulated cells near the tumour surface and the signal was transmitted deeper through cell-to-cell signalling events. However, this still does not explain how the initial 'plasma signal' penetrated through the intact skin barrier before reaching the tumour. Therefore, we hypothesised that apoptosis was induced by reactive species delivered by the He plasma jet into the tumour. This hypothesis was directly tested in the following experiment.

Tumours located at the rear of rodents (n = 5) were treated with the He plasma jet for 15 min (i.e. the same treatment time as figure 2). To measure ROS within the rodents, they were injected with Luminol, following a modified procedure first reported by Liu et al [89]. ROS react with Luminol to form a luminescent product underneath the skin. The Luminol-ROS reaction product lifetime is approximately 30 s. No luminescence was detected in the control group of rodents with the tumours treated with the control He gas flow (figures 3(A) and (C)). This result indicates that the He gas flow on its own does not deliver ROS into the tumours. After He plasma jet treatment, a strong luminescent signal was detected (figures 3(B) and (C)). This signal was statistically higher compared to the He gas flow control (n = 5,p < 0.05), indicating that ROS were evidently delivered through the tumours by the He plasma jet. This result was relatively surprising because it shows the He plasma jet delivered ROS across an intact epidermal layer that normally provides an effective barrier to, for example, harmful agents and moisture ingress.

From figure 3(B), (He plasma jet treatment for 15 min) a strong distribution of ROS at the target tumour site can be seen. And, whilst there was mice-to-mice variation, luminescence was more intense in the areas surrounding the point of He plasma jet contact (and not directly at the point of contact). This result is in good agreement with previous work (using a different He plasma jet and a gelatin target) [66]. The common feature is that micrometre- to millimetre-diameter He plasma jets deliver ROS onto the surface of tissue-like materials in a 'ring-shaped' pattern that can spread over an area greater than 10 mm in diameter, depending on the experimental conditions. Reviewing earlier work by Sakiyama et al, a potential explanation for the ringshaped distribution of ROS comes from a study of the ground state atomic oxygen generation within the tube of a He plasma jet [90]. In this study, it is shown using two-photon absorption laser-induced fluorescence spectroscopy that a He plasma needle discharge (operated at a flow rate of 1 slpm, i.e. the same flow rate used in this study) delivers ground state atomic oxygen in a ring-shaped distribution with a peak density of 10^{15} cm^{-3} [90].

Interestingly, the Luminol experiments also showed raised ROS levels in the mice far from the tumour site, with strong signals for ROS detected around the eyes and mouth region. This result indicates that ROS directly transferred through the skin barrier by the He plasma jet, or created downstream within the tissue fluid or tissue, were (in either case) subsequently systemically transported by the physiological fluid flow to distant sites in the mice.

With respect to clinical application, one issue of the mouse experiments is that the human skin barrier is much thicker. However, human skin was not available for these experiments. Consequently, the He plasma jet delivery of ROS through pig skin connected to a 1 mm layer of



Figure 2. Induction of apoptosis within a 2.8 ± 0.5 mm thick tumour on a live rodent after He plasma jet treatment. Full histological sections of TUNEL and H + E stained resected tumours are shown after treatment with the (A) neutral He gas flow and (B) He plasma jet. The region of the tumour that was in the direct line-of-sight of the He gas flow or He plasma jet is indicated by arrows. (C) Cells stained positive for TUNEL as indicated by the red arrows. (D) Cells stained positive for TUNEL were scored in the histological sections from the surface of the tumour (0 mm) to a maximum depth of 2.8 mm within the tumour after neutral He gas flow and He plasma jet treatments. (E) The total number of apoptotic cells as function of depth within the tumour. It can be seen that the He plasma jet induced apoptosis in a significant higher number cells compared to the He gas flow treatment (n = 5, p < 0.001).

sub-cutaneous fat was also measured after the same 15 min of He plasma jet treatment (figure 4(A)). Pig skin was chosen on the basis that it has similar physicochemical properties to human skin [91, 92]. Initially, UV-vis spectroscopy was employed for this experiment, as per the agarose tissue model experiments. However, interferences in the UV absorbance, presumably due to proteins from the pig skin, made the measurements unreliable. Therefore, the transport of ROS through pig skin was monitored using the ROS indicator DCFH-DA. As a control, it was shown that a 1 mM spot of H_2O_2 solution could not penetrate the pig skin after 15 min (figures 4(A) and (B)), indicating that the pig skin is otherwise an impermeable barrier to aqueous ROS (and by inference, also to RNS). After He plasma jet treatment (figure 4(A)), no visible damage was detected (by eye) to the pig skin, but ROS were readily delivered through the pig skin, indicated by a clear fluorescence increase from oxidised DCFH (figure 4(B)).

In order to further understand the role of the He plasma jet reactive species in the treatment of a cancer tumour, a simple experimental set-up was employed wherein a He plasma jet or neutral He gas jet (i.e. with the plasma off) were used to treat agarose tissue models placed on top of a cuvette filled with DI water (DI water provided a collection reservoir for the RONS transported through agarose). The transport of RONS and $O_2(aq)$ were measured in real-time by UV–vis spectroscopy of the DI water. The purpose of this set-up was to monitor the dynamics of the delivery of RONS subsurface and $O_2(aq)$ (to millimetre depths) that would likely apply to the live rodent tumour model. The electrical and optical property of the He plasma jet were measured in tandem with UV–vis spectra during the He plasma jet treatment of the DI



Figure 3. *In vivo* detection of ROS after He plasma jet treatment of a tumour on a live rodent. The control was the rodent treated with the neutral He gas flow with no applied voltage (i.e. with the plasma off). The spatial distribution pattern of luminescence observed in the rodents after treatment with the (A) neutral helium gas flow and with the (B) He plasma jet. (C) Total luminescence measured over the entire rodent after the treatments. A significant level of luminescence was detected in the rodents treated with the He plasma jet, whereas no luminescence was observed in the rodents treated with the neutral He gas flow (n = 5, p < 0.05).



Figure 4. The He plasma jet delivery of RONS through pig skin with 1 mm sub-cutaneous fat. The treatment time was 15 min. For the control, the same experiment was performed with He gas flow only (i.e. with the plasma off) or by incubating a 1 mM spot of H_2O_2 solution over the top of the skin for 15 min. The experimental set-up is shown in (A). The experimental results are shown in (B). Measurement was taken at $t = 30 \min (10 \min \text{He plasma jet treatment} + 20 \min \text{plasma and He gas flow off})$. The He plasma jet treatment resulted in a clear switch-on of DCFH fluorescence compared to the control. This indicates that RONS were readily transported through the pig skin by the He plasma jet treatment.

water (*direct treatment*) or the agarose tissue model (*through-agarose treatment*), as shown in figures 5(A)-(C). The set-up enables a better link between the He plasma jet properties to the therapeutic agents detected *in situ*. Potentially, this should aid in the development of new models for the prediction and analysis of plasma interactions with tissue [93–101].

The electrical and optical properties of the He plasma jet were characterised in order to understand how these plasma properties might influence the treatment of the cancer tumour (figure 6). The (A) driving voltage, (B) total current, $I_{\rm T}$, and the displacement current, $I_{\rm dis}$, and (C) discharge current, $I_{\rm D}$, are shown. The $I_{\rm D}$ of the He plasma jet showed two single peaks per period associated with the voltage rise and fall. The peak-to-peak amplitude and full-width-half-maximum of the positive and negative $I_{\rm D}$ were similar at around 0.7 mA and 10 μ s, respectively. The $I_{\rm D}$, was calculated by subtracting $I_{\rm dis}$, determined in the absence of the plasma discharge but with the applied voltage, from $I_{\rm T}$. From the voltage and $I_{\rm D}$ measurements, averaged input power, $\overline{P_{\rm in}}$, was calculated to be 0.25 W from equation (1):

$$\overline{P_{\rm in}} = f \int_0^T V(t) \times I_{\rm D}(t) \mathrm{d}t, \qquad (1)$$

where *f* is the frequency (=10 kHz) and *V* is the voltage (= 7 kV peak-to peak) (1).



Figure 5. (A) The UV-vis set-up to measure the real-time He plasma jet delivery of RONS into DI water directly (direct treatment) and (B) indirectly through an agarose target as surrogate of tissue (through-agarose treatment), and (C) the complete experimental set-up.

The typical optical emission spectrum of the He plasma jet between (D) 280-480 nm (top) and (E) 640-840 nm are also shown. From these spectra we can see signals from the excited He gas (He^{*}) and other species (N₂^{*}, O^{*} and the OH radical) that were generated when the plasma jet contacted the air. Many of these species will be highly reactive with short halflife and would be much more important in the direct treatment of cells or tissue. From the (F) fitted data, the plasma vibrational ($T_v = 0.27 \text{ eV}$) and rotational temperature ($T_r =$ 0.025 eV) were determined. These values were derived from the nitrogen gas (N₂) second positive system ($C^3\Pi_u$ -B³ Π_o) between 370 and 385 nm (2). Using the theoretical calculation of the N₂ second positive system [C³ Π_{μ} ($\nu' = 0, 1$) and B³ Π_{g} $(\nu''=2, 3)$], a best fit of the spectrum with a T_r of 0.025 eV was obtained. $T_{\rm r}$ is considered the gas temperature because of the rapid rotational relaxation through inelastic collisions between molecules and atoms [102]. The value of T_r $(0.025\,\text{eV})$ of N_2 molecules obtained in this study is in agreement with a previous calculation using the emission spectrum for the OH[•] [103]. Based upon the plasma electron temperature $(T_e) \approx T_v$, the T_v was estimated at 0.27 eV. However, this is only an approximation because T_v can be affected by inelastic collisions between the electrons and molecules [102, 104]. The much higher T_e of 0.27 eV compared to T_r of 0.025 eV is indicative of the non-equilibrium nature of the plasma. $T_{\rm r}$ was taken as the bulk plasma gas temperature and the jet was 'cool' to touch. Therefore, it is unlikely that the increased frequency in apoptosis within the tumour was caused by heating during He plasma jet treatment.

The high-energy components of the plasma are responsible for the creation of reactive species, including RONS that are relevant to the rest of this study. The T_v of 0.27 ev is ≈ 3000 K, which is sufficiently high to facilitate nitrogen–oxygen chemistry [105]. Excited state species, such as He^{*}, (seen in the optical emission spectroscopy, OES) may be responsible for energy transfer to create, for example, the highly reactive OH^{\bullet} and excited oxygen, O^* , (both seen in the OES). These highly reactive species are all short-lived and will only be relevant at the plasma-liquid/tissue interface [94]. The resultant species created in the DI water were measured by UV–vis.

Employing the set-up shown in figure 5, UV-vis was used to assess the delivery of RONS directly into DI water (utilised as surrogate of tissue fluid) and indirectly through agarose tissue models. The UV absorption spectra were recorded from 190 to 340 nm immediately after 15 min of treatment (t = 15 min) and at further time-points at t = 30, 45, and60 min (i.e. 15, 30, and 45 min after both the plasma and He gas flow were extinguished at t = 15 min), respectively. Direct treatment of DI water resulted in a single and prominent UV absorbance peak centred at 205.8 nm at t = 15 min(figure 7(A)). There was little further change in UV absorbance seen after the plasma and He gas flow were extinguished over the following period, t = 15-60 min. This contrasted markedly with the through-agarose treatment for a 2.5 mm thick agarose (figure 7(B)). At t = 15 min, the intensity of the main UV absorbance peak, centred at 194 nm, was lower. However, the UV absorbance continued to increase after extinguishing the plasma and He gas flow and eventually exceeded the intensity of the corresponding peak for direct treatment by t = 30 min.

In figure 7(C), the total UV absorbance between 190 and 340 nm, is plotted as a function time for direct and throughagarose treatments, with agarose thicknesses of 1.3, 2.5, and 5.8 mm. These thicknesses were chosen as they enable estimates of the likely minimum RONS concentrations delivered (by the He plasma jet) into the tumour as already discussed: i.e. through the top third (1.3 mm) and bottom third (2.5 mm) of the 2.8 ± 0.5 mm thick implanted tumours, as well as (5.8 mm) below these tumours. In this experiment, the total RONS and $O_2(aq)$ were taken from the total UV absorbance between 190 and 340 nm [71]. For the direct treatment, there was an almost immediate and monotonic increase in RONS before a clear inflection point at $t = 15 \min$ (marked solid arrow), that coincided with the extinguishing of the plasma and He gas flow. In the through-agarose treatments there was a time lag of minutes before the appearance of RONS at t = 6, 12, and 24 min through the 1.3, 2.5, and 5.8 mm thickagarose, respectively. These are marked with open arrows. Unlike direct treatment, the delivery rate of RONS into the DI water was not affected by extinguishing the plasma and He gas flow, with no inflection point observed at t = 15 min. As discussed below, the RONS concentrations were determined from the UV absorbance spectra [68-71]. For direct treatment, the RONS concentration increased in the DI water at a rate of 0.110 mg l^{-1} min⁻¹ between t = 1.5-16.5 min. For the through-agarose treatment, the delivery rate was reduced by increased agarose thickness to 0.132, 0.079, and $0.012 \text{ mg l}^{-1} \text{ min}^{-1}$ for 1.3, 2.5, and 5.8 mm thick agarose, respectively¹³. Close inspection of the agarose targets, following treatment with the He plasma jet, revealed an

¹³ These values were measured 15 min immediately after the lag time, i.e. between t = 6-21 min, t = 12-27 min and t = 24-39 min.



Figure 6. Electrical and optical characteristics of the He plasma jet. The He plasma jet was operated with a He flow rate of 1 lpm at a driving voltage of 7 kV (peak-to-peak) with a frequency of 10 kHz. (A) shows the driving voltage waveform, (B) total current and (I_T) and displacement current (I_{dis}) waveforms, and (C) discharge current (I_D). Inspection of the optical emission spectrum of the He plasma jet between (D) 280–480 nm and (E) 640–840 nm shows that the N₂ first negative and the N₂ second positive systems dominated the spectrum, with lower intensity signals from excited He (He^{*}) and the hydroxyl (OH) radical. Signals from He^{*} and excited oxygen (O^{*}) are seen more clearly between 640–840 nm. (F) The measured spectrum (open circles) is shown with the calculated spectrum (fitted line) from the spectrum simulator of the N₂ second positive system (kindly provided by Professor Hiroshi Akatsuka at Tokyo Institute of Technology).

indentation created at the point of He gas flow contact, presumably due to dehydration (supporting information, S2). But there was no evidence that He plasma jet treatment had created a hole in any of the agarose targets, through heating, gas flow or other processes, which would have affected the barrier properties of the target and provided a direct channel for the RONS to reach the DI water.

From the data in figure 7(C), it is clearly seen that in direct He plasma jet treatments the initial delivery of RONS into DI water was much faster when compared with throughagarose treatments for the period of plasma ignition (t = 0-15 min). Faster RONS delivery without the barrier can be explained by fast solvation of RONS from the gas phase into the DI water [94]. And, in the case of direct treatment the flux of highly reactive/short-lived species should also be considered, such as the He metastable, OH and atomic oxygen, which give rise to further RONS [54, 106]. In comparison, the agarose provided a barrier to the impinging plasma flux and slowed the transport (and solvation) of RONS into the DI water. The time-lags seen (increasing with agarose thickness) are presumed to result from RONS transport by slower molecular processes through the targets and into the DI water [69, 71]. These data suggest that the plasma jet first creates a reservoir of RONS and $O_2(aq)$ in the agarose that is then subsequently eluted from the agarose into the DI water for at least up to 45 min after the plasma and He gas flow are extinguished. Eventually, the total UV absorbance in the DI water treated indirectly through 1.3 and 2.5 mm thick agarose exceeds that seen in the direct treatment. However, insight into how this translates to specific RONS and their respective concentrations requires more detailed analyses of the raw spectra in figures 7(A) and (B).

The major long-lived RONS generated in DI water by plasma have been shown to be H_2O_2 , NO_2^- and NO_3^- [107–111]. Strictly, NO_2^- and NO_3^- are not RONS. However, although the reliability remains questionable [112], measures of NO₂⁻ and NO₃⁻ are often taken as surrogates of NO production in whole blood or plasma [113]. NO_2^- and NO_3^- can also be end products of highly-reactive ONOO⁻. Consequently, UV absorption spectra of neat solutions of these RONS, as well as $O_2(aq)$, were measured (figures 8(A)–(D)). These spectra informed the subsequent peak-fitting of H_2O_2 , NO_2^- , NO_3^- , and $O_2(aq)$ components in the UV absorbance spectra recorded from the DI water after either direct or through-agarose treatment, as shown in figures 8(E) and (F). In both cases, the treatment regime was the same, i.e. plasma jet treatment for 15 min, followed by 45 min incubation, with the plasma and He gas flow extinguished, before a UV absorption spectrum was recorded at t = 60 min. In a previous publication, the UV-vis automated curve-fitting routine was shown to accurately measure RONS in DI water containing a mixture of known concentrations of H₂O₂, NO₂⁻, NO₃⁻, and O₂(aq) [68]. A major advantage of the UV-vis curve-fitting routine is its capability to make multiple, real-time measurements of RONS and $O_2(aq)$.

Based upon the peak-fitting in figures 8(E) and (F), both direct and through-agarose treatments (at t = 60 min) produced a RONS concentration profile in the descending order: H₂O₂ > NO₂⁻ + NO₃⁻, which follows the order previously



Figure 7. Time-dependent changes in the UV absorption profile and total UV absorbance of DI water treated directly and indirectly through agarose with the He plasma jet. UV absorbance spectra are shown for (A) direct and (B) through-agarose He plasma jet treatments at t = 15, 30, 45, and 60 min. The plasma and He gas flow were switched off at t = 15 min. The agarose thickness was 2.5 mm. (C) Time-dependent changes in the total UV absorbance between 190 and 340 nm in DI water with He plasma jet treatments for direct and through-agarose treatments (with agarose of thicknesses of 1.3, 2.5 and 5.8 mm). The open arrows indicate positive inflection points in the curves, where there was a marked increase in RONS, and the solid arrows indicate negative inflection points in the curves, where rate of RONS generation slowed. The green shaded region corresponds to the He plasma jet treatment time of 15 min.

reported for the He plasma jet treatment through gelatin and agarose tissue models [65, 68]. At t = 60 min in figures 8(E) and (F), the major differences between direct and through-agarose treatments were: higher quantities of RONS generated for the through-agarose treatment; virtually no NO₂⁻ detected in DI water for the through-agarose treatment; and the DI water was de-oxygenated by direct treatment, but conversely oxygenated by the through-agarose treatment.

The curve-fitted data are plotted as function of time to highlight the time-dependent changes in the concentrations of H₂O₂, NO₂⁻, NO₃⁻ and O₂(aq) in the DI water up to 60 min (figures 8(G)–(J)). At t = 15 min both the plasma and He gas flow were extinguished. Direct treatment resulted in an immediate and monotonic increase in H₂O₂, NO₂⁻ and NO₃⁻ up to t = 15 min. After extinguishing the plasma jet, no further increase in these molecules was detected. The final RONS concentrations in descending order were: $H_2O_2 > NO_2^- > NO_3^-$. The final H_2O_2 concentration was 1.03 mg l⁻¹ ($\approx 30 \,\mu$ M), which is most likely to be toxic to cells [2]. An additional small intensity peak centred at 265 nm was sometimes seen in the UV absorption spectra; this peak was excluded in the curve-fitting but is discussed in the supporting information, S3.

An interesting result is the de-oxygenation of DI water by direct treatment. Over the 15 min when the plasma jet was on, the O₂(aq) decreased, with the He gas flow purging dissolved gases out of the DI water. The maximum loss of O₂(aq) was 6.57 mg l^{-1} observed at completion of treatment (t = 15 min). After t = 15 min, the O₂(aq) concentrations



Figure 8. Concentrations of specific RONS in DI water treated with the He plasma jet directly and through agarose, as determined with the automated UV–vis curve-fitting routine. Reference UV absorbance spectra for neat solutions of 1 mg l^{-1} (A) H₂O₂, (B) NO₂⁻, (C) NO₃⁻ and (D) O₂(aq), respectively, dissolved in DI water. These peaks were used to determine the concentrations of RONS in the DI water after He plasma jet treatments with an automated curve-fitting routine. Curve-fitted UV absorption spectra after (E) direct and (F) through 2.5 mm thick agarose He plasma jet treatment of DI water. The He plasma jet treatment was set at *t* = 15 min and the spectra were recorded at 60 min (i.e. 45 min after the plasma and He gas flow were switched off). The change in concentrations of (G) H₂O₂, (H) NO₂⁻, (I) NO₃⁻ and (J) O₂ are plotted as function of time to illustrate their time-dependent change during and post He plasma jet treatment. In (G)–(J), the He plasma jet treatment time of 15 min is indicated by the shaded green region, and the positive and negative inflection points on the graphs are indicated by open and solid arrows, respectively.

begins to recover, as oxygen from the ambient air solvated into the DI water. But, at t = 60 min, the O₂(aq) concentration was still below the ca. 8 mg l⁻¹ for untreated DI water.

The delivery rate of RONS and changes in $O_2(aq)$ are markedly affected by the agarose targets and by target thicknesses. The delivery rate of H₂O₂ into DI water directly versus through-agarose, was not affected by the 1.3 mm thick agarose tissue model during the 15 min of plasma jet treatment. But for the through-agarose treatment, the concentration of H₂O₂ in DI water continued to increase after the plasma and gas flow were both extinguished, and at t = 60 min at $152.4 \pm 30.5 \,\mu\text{M}$, exceeded the direct treatment of DI water (34.1 \pm 4.0 μ M). For the 2.5 mm thick agarose target, a time-lag of 16.5 min was observed before the H₂O₂ increased in the DI water; i.e. H₂O₂ appeared in the DI water about 1.5 min after the plasma and gas flow had been extinguished (positive inflection points are shown by open arrows). The H_2O_2 continued to rise underneath the 1.3 and 2.5 mm thick agarose over the 60 min period; but at reduced rates from approximately $t = 28 \min$ and $t = 43 \min$ for the 1.3 and 2.5 mm thick agarose, respectively (negative inflections points are shown by solid arrows). At 60 min, the concentration of H₂O₂ delivered through the top 2.5 mm of the agarose exceeded 3.66 mg l^{-1} (108 μ M); a concentration that would be highly cytotoxic [2]. The 5.8 mm agarose target presented a more substantial barrier to H₂O₂. A time-lag was not obvious with this target, with only a gradual rise in the H_2O_2 concentration of less than 1 mg l^{-1} (30 μ M) observed at t = 60 min.

As noted, NO_2^- was only detected in the direct treatment of DI water. This result is perhaps not too surprising given the longer half-life of NO_3^- and the ready conversion of NO_2^- to NO_3^- . For example, in whole blood, NO_2^- has been shown to be readily oxidised to NO_3^- [113]. For NO_3^- there were time-lags of 6, 7.5, and 24 min before NO_3^- began to appear in the DI water underneath the 1.3, 2.5, and 5.8 mm thick agarose, respectively. The concentration of NO_3^- in the DI water began to plateau at t = 25.5 and 40 min underneath the 1.3 and 2.5 mm thick agarose, respectively. For the 5.8 mm thick agarose, the concentration of NO_3^- in DI water did not plateau but continued to increase up to t = 60 min.

In standard DI water, the measured $O_2(aq)$ concentration is ca. 8 mg l⁻¹; for the through-agarose treatment after 60 min the concentration of $O_2(aq)$ had increased by 6.60 mg l⁻¹ for the 1.3 thick mm agarose, whilst for the direct treatment, at the same time point the $O_2(aq)$ concentration was reduced by 1.00 mg l⁻¹ (but as noted above, this followed a significant reoxygenation after extinguishing of the plasma). These data are consistent with earlier work that showed He plasma jet treatment through soft, hydrated tissue models, increased the $O_2(aq)$ concentration in a solution below the tissue model, but direct He plasma jet treatment de-oxygenated the solution [68, 70]. Again, with agarose, a time-lag is seen before the $O_2(aq)$ increased in the DI water. There was an initial delay of

Table 1. Micromolar concentrations of specific RONS estimated from peak-fitting of UV–vis data. He plasma jet treatments were performed through agarose of 1.3, 2.5, and 5.8 mm thicknesses or directly into DI water (Direct) for 15 min. UV–vis measurements were made (A) immediately after treatment (t = 15 min), and after (B) 15 min (t = 30 min) and (C) 45 min (t = 60 min).

	RONS type (μ M)	H_2O_2	NO_2^-	NO_3^-	O ₂	Total RONS (μ M)
A	1.3 mm	31.2 ± 10.6	0.0 ± 0.5	0.8 ± 0.5	46.8 ± 33.6	31.6 ± 11.6
	2.5 mm	3.2 ± 2.9	0.0 ± 0.1	0.4 ± 0.1	2.8 ± 5.3	3.4 ± 3.2
	5.8 mm	4.2 ± 2.2	0.0 ± 0.0	0.0 ± 0.0	2.7 ± 2.8	4.14 ± 2.2
	Direct	30.2 ± 3.9	5.6 ± 0.2	2.6 ± 0.1	-205.3 ± 6.6	38.4 ± 5.3
В	1.3 mm	109.9 ± 29.1	0.0 ± 0.2	1.3 ± 0.2	184.4 ± 34.6	110.9 ± 29.5
	2.5 mm	45.8 ± 13.3	0.0 ± 0.3	1.6 ± 0.13	140.0 ± 22.4	47.2 ± 13.7
	5.8 mm	4.3 ± 2.1	0.0 ± 0.0	0.2 ± 0.1	6.5 ± 3.5	4.5 ± 2.2
	Direct	32.0 ± 4.7	6.5 ± 0.1	2.4 ± 0.0	-107.3 ± 4.6	40.9 ± 4.9
С	1.3 mm	152.4 ± 30.5	0.1 ± 0.3	1.2 ± 0.2	206.1 ± 32.8	153.7 ± 31.0
	2.5 mm	107.6 ± 13.1	0.0 ± 0.6	1.9 ± 0.1	224.6 ± 19.2	109.6 ± 13.7
	5.8 mm	16.5 ± 1.1	0.2 ± 0.1	1.8 ± 0.3	52.7 ± 1.9	17.9 ± 1.4
	Direct	34.1 ± 4.0	6.7 ± 0.1	2.4 ± 0.0	-31.3 ± 4.7	43.1 ± 4.0

9 and 15 min for the 1.3 and 2.5 mm thick agarose, respectively. The O₂(aq) concentrations continued to rise after the plasma and gas flow were extinguished. A plateau in the O₂(aq) concentration in DI water was reached at t = 25.5 and 34.5 min for 1.3 and 2.5 mm thick agarose, respectively. Only a small, gradual rise in the O₂(aq) concentration in the DI water of less than 1.7 mg l⁻¹ was observed underneath the 5.8 mm thick agarose at t = 60 min.

The data in figure 8 highlight the complexity in the He plasma jet delivery of RONS and O2(aq) into soft hydrated materials. Dynamic changes in RONS and O2(aq) occur in DI water during and post-plasma treatment for through-agarose treatments. If the DI water and agarose tissue model represents tissue fluid and tissue, respectively, then considering two time-points at the completion of treatment (t = 15 min) and 20 min after treatment (t = 35 min, which corresponds to the time-point of the Luminol live rodent experiment already discussed), it can be seen that the RONS and $O_2(aq)$ concentrations are different within the tissue's top 5 mm. At $t = 15 \text{ min}, \text{H}_2\text{O}_2$ has penetrated tissue to a depth of 1.3 mm (albeit very rapidly), whereas NO_3^- has penetrated deeper to 2.5 mm. And, marginal oxygenation of the DI water is seen only with the thinnest 1.3 mm thick agarose. But, at t = 35 min, the concentration of H₂O₂ with agarose in descending order is: 1.3 > 2.5 > 5.8 mm, whilst at the same time-point, the NO_3^- concentration with agarose in descending order is: 2.5 > 1.3 > 5.8 mm. Although this last observation might seem surprising, it can be explained by examining the time-point at t = 60 min, where the NO₃⁻ concentration with agarose in descending order is: $2.5 \approx 5.8 > 1.3$ mm. This result would imply that postplasma jet treatment, NO_3^- is more efficiently transported to a greater depth in the tissue models than H_2O_2 .

Overall, these results indicate that soft, hydrated material, such as living tissue, can act as a reservoir for (the storage of) RONS and $O_2(aq)$ during He plasma jet treatment, and post He plasma jet treatment these RONS and $O_2(aq)$ are gradually transported deeper into tissue. At t = 60 min, the total RONS (figure 7(C)) has plateaued in the DI water below 1.3 and 2.5 mm thick agarose (but not yet at 5.8 mm). The values in

figures 8(G)–(J) represent the concentrations of RONS and $O_2(aq)$ that have eluted from the agarose into the DI water underneath. Recalling that the agarose comprise in large part water, it can be assumed these values represent the minimum concentration of each of these specific RONS and $O_2(aq)$ within the agarose. Taking the data from figures 8(G)–(J), table 1 shows estimate values of the minimum concentrations of each of the RONS at three different depths at the t = 15, 30, and 60 min time points.

Focusing upon the trends in table 1, clear differences can be seen in the concentration gradients of RONS and O₂(aq) within the agarose at the t = 60 min time point. H₂O₂ and $O_2(aq)$ are highly concentrated within the top 2.5 mm, but by 5.8 mm their concentrations have fallen to less than one fifth. However, even at this depth, the concentration of H_2O_2 at a value of 16.5 μ M significantly exceeds the 1–5 μ M concentration range that Forman et al [114] considered normal for blood and plasma, and is becoming closer to those measured in chronic inflammation (30–50 μ M) [114]. NO₃⁻ is more evenly distributed over the top 5.8 mm in the range of 1.2–1.9 μ M; but in contrast to H₂O₂, this range is somewhat lower than those determined in plasma (in 26 healthy volunteers $NO_2^- = 1.3-13 \,\mu M$ and $NO_3^- = 4.0-45.3 \,\mu M$) [113]. In the top 1.3 mm, the concentrations of H_2O_2 increase post He plasma jet treatment from t = 15 to 60 min from 31.2 μ M to 152.5 μ M. Whilst this may in part result from the time-dependent transport process of H₂O₂ through agarose into the DI water, the initial t = 15 min value is already comparable to that obtained by the direct He plasma jet treatment of DI water. We therefore strongly suspect that the He plasma jet, in addition to delivering H_2O_2 into agarose, created more complex RONS (by reaction with the agarose) that were subsequently converted to H₂O₂. In previous work, it has been noted that using more complex biological media in place of DI water (i.e. PBS with added protein), it is possible to create 'secondary' RONS; it is suspected that these arise from the reaction between dissolved RONS and protein to yield alkyl radicals [74]. These in turn react with $O_2(aq)$ to produce alkylperoxyl radicals (ROO[•]) that have an extended lifetime. The formation of stable protein hydroperoxides

provides (upon their decomposition) a reservoir of smaller messenger RONS over an extended period of time from hours to perhaps days after the initial He plasma jet treatment. Although agarose does not contain protein, H_2O_2 is known to oxidize carbohydrates in a process that is rapidly accelerated by trace amounts of ferrous ions [115]. And polysaccharides, e.g. agarose, are known to undergo oxidative depolymerisation that could potentially generate smaller reactive fragments [116].

4. Discussion

The use of agarose (and gelatin in previous work) tissue models have shown two independent and potentially equally important events occurring during He plasma jet treatment to depths on the millimetre scale-length. The *first* is the delivery and transport of RONS (and other molecules) into tissue. Herein, a He plasma jet directly delivered H₂O₂, NO₂⁻, and NO_3^- into DI water. There is an abundance of literature on the biological roles of H₂O₂, and as noted, it is ordinarily present in blood or plasma in the concentration range of $1-5 \mu M$. The delivery of exogenously created H₂O₂ has the potential to play multiple therapeutic roles. It has been shown in that H_2O_2 below 10 μ M can stimulate cell proliferation but at concentrations above $10 \,\mu\text{M}$, H₂O₂ can be cytotoxic [2]. Therefore, it is possible that the high concentrations of H_2O_2 potentially delivered into the tumour on the rodent by the He plasma jet, could be responsible for apoptosis throughout the entire depth of the tumour.

The plasma delivery of NO is often emphasised, as it is implicated as a key signalling molecule in a number of biological processes [13]. Yet, the lifetime of NO is relatively short and the breakdown of NO in aqueous solutions yields mainly NO₂⁻ [117]. NO₂⁻ (and NO₃⁻) could also have important role in cancer therapeutics, and there is now a new emerging school of thought that NO₂⁻ and NO₃⁻ metabolism occurs (in blood and tissues) to give NO and other bioactive nitrogen oxides [86]. Particularly, in hypoxic tissue NO₂⁻ and NO₃⁻ are viewed as possible sources of NO_x in times of metabolic stress, with downstream impacts on blood vessel dilation and intracellular signalling transmission, ion channel conduction, glycogen decomposition and apoptosis [86, 118]. Therefore, it is also possible that NO_x species stimulated apoptosis within the tumour in this study.

The *second* is the oxygenation of tissue (as opposed to oxidation) by the delivery and transport of $O_2(aq)$. The delivery of $O_2(aq)$ by a He plasma jet could counteract hypoxia. Hypoxia has been reported to increase the resistance of cancer cells (within a solid tumour) to radiation and cytotoxic drugs [119]. The experiments with the agarose tissue models indicate that the He plasma jet treatment will raise the local oxygen tension of the irradiated area. Whilst in this study the oxygenation of the tumour in the rodent experiment was not measured, previously Collet *et al* have shown that a He plasma jet induced tissue oxygenation in live mice [64].

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This is supported by Keidar *et al* who have shown that ROS levels were elevated within the top third (to a depth of 3 mm) of a tumour after plasma jet treatment [120]. Whilst beyond the scope of this study, tissue oxygenation by plasma jets (from RONS and $O_2(aq)$, respectively) might be a potential strategy to assist in the destruction of cancer cells within solid tumours when preceding radiotherapy, by raising local oxygen tension.

In the context of developing cold atmospheric plasma as a standalone technology for the treatment of tumours, it is often stated that at least some types of cancer cells are more susceptible to attack from RONS than healthy cells because: (1) cancer cell membranes have less cholesterol making their membranes 'barrier' more susceptible to lipid peroxidation, which leads to pore formation and higher ingress of RONS [121], (2) cancer cells exhibit increased intrinsic ROS-stress, supposedly making the cells more vulnerable to oxidative damage [122, 123], and (3) owing to their proliferative nature, cancer cells spend a greater percentage of time in the S-phase of the cell cycle (i.e. DNA synthesis stage) making the new DNA more susceptible to oxidative damage [58]. These reasons have all been put forward to explain why He plasma jets selectively target the destruction of cancer cells compared to healthy cells, as summarised in the following reviews [47, 56, 120, 124]. The data in this study provides at least some evidence that plasma-generated RONS and $O_2(aq)$ are transported into tissue to reach cells that are deeper within tumours.

It is also worth commenting upon the limitations of the method, and therefore the potential to improve upon the simple agarose tissue model. First, the experiment is static (i.e. there is no physiological fluid flow). In the rodent data, it can be seen that Luminol is activated at sites very distant to the target tumour site that was directly exposed to the He plasma jet. By introducing fluid flow into the experiment, perhaps with a small pumping system, it would be interesting to see how far RONS species persist from the He plasma jet treatment site. Second, agarose is devoid in biological material, but it is possible to add biomolecules to mimic different tissues [125], or to incorporate live cells into the agarose matrix to mimic 3D tumours [126, 127].

Whilst it is shown that the epidermal barrier (pig or rodent skin) does not appear to present a significant obstacle to RONS transport, which is consistent with studies from other researchers [67, 73, 77], there is currently no firm explanation for this observation. It is possible that strong electric charges and fields generated during the operation of the He plasma jet might facilitate the transfer of RONS into the top few surface layers of the tissue [66, 67]. The neutral He gas flow is likely to be transmitted through the tissue, at least in the uppermost surface layers [128]. Strong coupling between charged species in the plasma jet to the neutral He gas could assist the delivery of RONS into the tissue [129, 130]. Recently, it was shown that a relatively small electric field of 20 V cm^{-1} can significantly enhance the delivery of RONS from an air surface plasma into gelatin,

which was used as a surrogate of tissue [67]. This strongly supports the claim that electric fields might assist the He plasma jet delivery of RONS into tissue.

The deeper penetration of UV photons could also help explain some tissue subsurface effects. For example, Attri *et al* have shown that an argon (Ar) plasma jet can generate 0.3×10^{16} cm⁻³ hydroxyl radicals (OH[•]) at a depth of 4 mm into DI water through UV photolysis [131]. This density was above the threshold required for inducing apoptosis in H460 lung cancer cells following one day incubation after the Ar plasma jet treatment.

In order to further develop safer and more effective plasma technologies for medical applications such as cancer therapy, it is important to understand the RONS (rates and doses) and $O_2(aq)$ that are delivered into tissue. RONS can potentially be cytotoxic and genotoxic within tissue, including even less reactive molecules such as H₂O₂, and at relatively low concentrations of 10 μ M [14]. This is advantageous for killing cells, e.g. in this study for killing cancer cells within a tumour. But the imperative should be to focus the delivery of RONS only to the diseased tissue and limit the excessive delivery of RONS to the healthy tissue; this would improve the safety of the plasma therapy. Therefore, this study is important in the context that it addresses (in part) the need to elucidate what RONS are delivered into tissue, as well as their delivery rate and penetration depth within tissue. The methodology presented in this paper can be readily developed to provide a more quantitative understanding of RONS and $O_2(aq)$ delivery into tissue to inform the effective and safe treatment of diseased tissues such as cancer tumours.

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