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The assessment of cold atmospheric plasma treatment of DNA in synthetic models of tissue fluid, tissue and cells

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Abstract

There is a growing literature database that demonstrates the therapeutic potential of cold atmospheric plasma (herein referred to as plasma). Given the breadth of proposed applications (e.g. from teeth whitening to cancer therapy) and vast gamut of plasma devices being researched, it is timely to consider plasma interactions with specific components of the cell in more detail. Plasma can produce highly reactive oxygen and nitrogen species (RONS) such as the hydroxyl radical (OH•), peroxynitrite (ONOO−) and superoxide (O2−) that would readily modify essential biomolecules such as DNA. These modifications could in principle drive a wide range of biological processes. Against this possibility, the reported therapeutic action of plasmas are not underpinned by a particularly deep knowledge of the potential plasma-tissue, -cell or -biomolecule interactions.

In this study, we aim to partly address this issue by developing simple models to study plasma interactions with DNA, in the form of DNA-strand breaks. This is carried out using synthetic models of tissue fluid, tissue and cells. We argue that this approach makes experimentation simpler, more cost-effective and faster than compared to working with real biological materials and cells. Herein, a helium plasma jet source was utilised for these experiments. We show that the plasma jet readily induced DNA-strand breaks in the tissue fluid model and in the cell model, surprisingly without any significant poration or rupture of the phospholipid membrane. In the plasma jet treatment of the tissue model, DNA-strand breaks were detected in the tissue mass after pro-longed treatment (on the time-scale of minutes) with no DNA-strand breaks being detected in the tissue fluid model underneath the tissue model. These data are discussed in the context of the therapeutic potential of plasma.

Szili and Gaur are equal co-first authors.
Keywords: plasma jet, DNA, molecular beacon, tissue model, synthetic cell model, phospholipid vesicles, plasma medicine

Supplementary material for this article is available online

(Some figures may appear in colour only in the online journal)

1. Introduction

There is a high degree of optimism that cold atmospheric plasma technologies will develop to significantly aid in the treatment of diseases, e.g. chronic wounds and cancers. It has been shown through in vitro cell studies and in vivo animal experiments that cold atmospheric plasma, herein referred to as plasma, might accelerate wound healing by stimulation of cell growth and migration [1–11], and also decontaminate infected wounds through targeted destruction of bacterial cells [12–22]. Similar results have been obtained in clinical studies where the efficacy of plasma in wound decontamination and healing has been demonstrated [23–26]. Apart from wounds, there is a rapidly growing interest in the use of plasma in cancer therapy. It has been demonstrated through in vitro cell studies that plasma can destroy cancer cells through various molecular pathways [27–32]; and it has been shown in vivo that plasma can be used to shrink tumours in rodents [33–35]. A clinical study has shown that plasma treatment may benefit cancer patients through pain relief, reduction in microbial load, partial tumour remission and improved wound healing [36]. To date there have been no reports of significant adverse effects from the clinical use of plasma; although plasma treatment has been reported to induce mild skin irritation, swelling, inflammation and superficial burns [23, 33, 36, 37]. And no significant adverse effects from plasma have been seen in patients following longer term clinical observations 12 months post plasma treatment [38].

The mechanism of plasma intervention in biology and medicine is thought to be mainly governed by reactive oxygen species (ROS) and reactive nitrogen species (RNS), or collectively RONS [39–41]. Experimentally, using models of tissue fluid, tissue and cells, it has been shown that plasma-generated RONS are not only delivered into tissue fluid but can also potentially be delivered deep within a tissue mass and across a phospholipid membrane into cells [7, 42–53]; computer simulations corroborate these experimental observations [54–61]. In the physiological environment, RONS regulate key biochemical pathways, inducing chemical and physical changes in cells [39, 62]. Where plasma-derived RONS intervene in biological processes is now a topic of great interest [39].

In cancer therapy, it has been argued that plasma-generated RONS detrimentally affect cancer cells whilst not damaging healthy cells due to differences in membrane composition and the molecular machinery between cancer and healthy cells. Arguments have been advanced that include: (1) the reduced cholesterol content in cancer cell membranes make these membrane more susceptible to pore formation, which enables a higher ingress of RONS that increases oxidative stress [55]; (2) the elevated intrinsic oxidative stress in cancer cells increases their vulnerability to oxidative damage [29]; and (3) the higher proliferation rates in cancer cells makes new DNA formed during the S-phase of the cell cycle more susceptible to oxidative damage [35]. In wound decontamination, the greater susceptibility of bacterial cells to plasma induced oxidative damage could arise from the increased surface area:volume ratio of these prokaryotic cells when compared to eukaryotic cells [46]; an increase in surface area:volume would lead to higher concentrations of RONS delivered into prokaryotes with a concomitant increase in oxidative stress.

The cell stimulatory effect of plasma has also been explained by the plasma delivery of RONS to tissue fluid, tissue and cells [6, 7]. Generally, if the dose or rate of delivery (flux) of RONS is kept low enough it is argued that RONS enhance cell proliferation. But, if the dose/flux of RONS continues to increase, it will almost certainly cause significant cell damage, eventually leading to cell death. In skin cells it has been shown that plasma can have beneficial or deleterious effects depending on the dose and flux of RONS [6, 7]. The stimulatory effect of plasma at low RONS doses and detrimental effect at high RONS doses have been described in terms of the principle of ‘hormesis’ [7, 63, 64]; a term commonly used for medical drugs where low doses are beneficial but high doses are not [65].

In the clinical use of plasma, the safety level (dose) for each different component of plasma such as RONS, neutral atoms and molecules, electrons and UV radiation, needs consideration in order to prevent potential (longer term) damaging effects. The safety requirements for plasma devices intended for clinical use have been discussed in a review by Heinlin et al [66]. Consideration has included electrical, UV, and reactive species components of plasma and the transmission of electrical current through skin. Plasma devices for medical use, such as the MicroPlaSter, have been reported to meet these requirements [66]. In addition, the inherent electric fields of plasma devices are expected to be relatively high and in the order of kV cm$^{-1}$ for some plasma devices [67]. Electric fields may play an important role in the plasma delivery of RONS through tissue [50, 51] and cell membrane [54] barriers. Consequently, electric field also requires careful consideration in the clinical application of plasma because of its potential to disrupt the integrity of tissue and cell membrane barriers.

However, the therapeutic action of plasma still relies on poorly understood cellular and tissue modifications, although some insights have emerged in recent years [41, 68, 69]. The central role for RONS now appears well established, but the mechanisms by which plasma-generated RONS affect cellular processes is still a topic of intense study and uncertainty. For
example, in many cases, the biological effects of plasma-generated RONS are likely to act through intermediate chemical species. This conclusion is drawn from an elementary consideration of the short lifetimes and diffusion distances of highly-reactive RONS when compared to the likely time and distance required for these RONS to reach cells in situ within tissue fluid and tissue, or even in in vitro cell culture media.

Although the safety requirements for the clinical use of plasma are being addressed in detail, a more holistic understanding of the nature of the possible damaging effects of plasma exposure is arguably still required in order to progress 'plasma medicine' laboratory research into the clinic. There is no question that the biological effects of plasma exposure are related to the intensity (power level) of the plasma and the time of plasma exposure. The precise nature of the 'biological plasma dose' is not well understood. But if the plasma treatment is too intense or too long, cells and tissue will be damaged or killed. For this reason, plasma devices approved for clinical use are operated within well-defined and carefully tested operational protocols. For example, the kINPen MED argon plasma jet source has been shown to be non-mutagenic when tested using in vitro ISO protocols [70] and in vivo tests with chicken embryos [71]. Bekeschus et al concluded that use of the kINPen MED for wound healing ‘does not pose any health risks in humans with regard to UV exposure, thermal damage, tissue toxicity, or mutagenicity’ [72].

Nevertheless, confidence in knowing the parameters of safe operation does not provide any insight into the possible mechanisms by which plasma exposure could cause significant cellular damage, including DNA damage. And, whilst no significant short or longer term adverse side-effects from plasma have been reported to date, it is important to continually review the safety of plasma. In the 2012 international ‘Plasma Roadmap’, Kroesen used an analogy with plasma etching to explain the importance of addressing the safety issues of plasma in medicine [73]:

‘The semiconductor industry was using plasma etching on very large scales well before a much better understanding was established. All that could go wrong is a less favourable process performance. The case of plasma medicine is totally different: human beings are at stake’.

Since the breadth of applications promoted for plasma ranges from the benign, e.g. in cosmetics such as teeth whitening and wrinkle treatment to the destruction of tumours in cancer therapy, it is reasonable to assume that there must be a graduated scale of risk:benefit that warrants consideration. As a simple analogy (that should not be pushed too far), a small exposure to x-rays is acceptable for imaging teeth and should do no harm if performed correctly, whereas higher doses that cause significant cell damage are utilised (specifically for this reason) in targeted cancer therapy.10

Amongst the RONS that may be generated by plasmas in tissue fluid and tissue are the hydroxyl radical (OH\(^*\)) and superoxide (O\(_2^−\)) [74] and peroxynitrite (ONOO\(^−\)) [75]. These RONS readily induce DNA-strand breaks [76, 77], and could explain plasma induced single- and double-strand DNA breaks in vitro [78–82]. In this study, we establish a methodology to assess plasma induced DNA-strand breaks in tissue fluid, tissue, and in cells. Rather than using real biological materials and cells, that would be expensive and time-consuming to work with routinely, and would significantly complicate the interpretation of any data, DNA-strand breaks are assessed in simple models of tissue fluid, tissue and cells. The main purpose of the tissue model we employ is to provide a barrier, similar to that encountered when plasma contacts any human tissue. The models utilised in these experiments are:

- **DNA**: Plasma induced DNA-strand breaks in the tissue fluid, tissue or cell models are assessed with a molecular beacon (MB) as previously described [80]. The MB is an oligonucleotide comprising a single-strand loop and double-strand stem structure. At the end of the stem is a 5′-fluorescent moiety and a 3′-quenching moiety. Single- or double-strand DNA breaks in the MB can result in separation of the quencher and fluorophore leading to switch-on of fluorescence. See figures 1(a)–(d).
- **Tissue fluid**: A 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (herein referred to as HEPES), which mainly comprises water, is used as a substitute for tissue fluid. HEPES is commonly used in cell cultures because of its excellent buffering capacity. See figure 1(a).
- **Tissue**: Gelatin is used as a substitute for the tissue barrier. Gelatin consists of collagen, one of the most abundant extracellular proteins, and provides a barrier to topically administered hydrogen peroxide (H\(_2\)O\(_2\)) solution (and by inference other RONS) [50]. See figures 1(b) and (c).
- **Cells**: Phospholipid vesicles are used to mimic the barrier of the phospholipid membrane of real cells. Reporter molecules can be packaged into the vesicles to analyse different processes such as RONS ingress and membrane permeability [46, 48, 49]. See figure 1(d).

The data are discussed in the context of obtaining a better understanding of plasma interactions with biological systems and the potential safety risks that could arise in the context of DNA-strand breaks. In addition, a simple method is developed to limit DNA-strand breaks whilst enabling the plasma delivery of longer-lived RONS. Specifically, we consider the delivery H\(_2\)O\(_2\). This method involves placing a hydrogel dressing in-between the plasma jet and the target liquid: the hydrogel acts as a ‘RONS filter’ enabling the rapid delivery of H\(_2\)O\(_2\) (and other longer-lived RONS) whilst preventing the delivery of shorter-lived RONS to the target.

2. Experimental

2.1. Plasma jet

The plasma source utilised was a ‘plasma jet’ configuration. The plasma jet comprised a 150 mm long glass tube, tapered from an

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10 We note that the application of x-rays cannot exert a beneficial biological response because x-rays ionise cells. However, treatment with plasmas can be used to stimulate cells (e.g. proliferation or migration) because plasmas do not ionise cells.
inner diameter of 4 mm to 650 \( \mu \text{m} \) at the nozzle. A 15 mm long external ring copper electrode, wound onto the glass tube at a distance of 40 mm from the end of capillary, served as the powered electrode. The plasma jet was used in a single electrode configuration with no designated grounded (second) electrode. The helium (He) gas (BOC, high purity grade) flow through the glass tube was controlled at 0.05 and 0.5 standard litres per minute (slpm) with a digital mass flow controller (Apex, 0–2 slpm flow range).

A sinusoidal voltage of 10 kV\(_{p-p}\) (peak-to-peak) at 30 kHz was applied to the external electrode with a PVM500 power supply (Information Unlimited). A 10 mm thick polytetrafluoroethylene (PTFE) housing was used to shield the high voltage electrode for safety. The treatment distance was 1 mm between the nozzle of the plasma jet assembly and the top of 96-well plate, or tissue model or hydrogel dressing. A photograph of the plasma jet device is shown in figure 2(a) and a diagrammatical representation of the plasma jet device with the experimental parameters in figure 2(b).

2.2. Reagents

The details of the reagents used in this study are shown in table 1. All reagents were used without further purification.

2.3. HEPES tissue fluid model

The following reagents were dissolved in 100 ml of water:
- HEPES—238.3 mg
- NaCl—624 mg
- NaOH—22.4 mg
- EDTA—29.22 mg

The solution was adjusted to pH 7.4 with drop-wise addition of 10 mM NaOH. The solution was filtered through a 0.2 \( \mu \text{m} \) syringe filter.

2.4. Tris-base buffer

Concentrated 1 M Tris-base buffer was prepared by dissolving 12.11 g Tris-base in 80 ml water. The pH was adjusted to 7.6 with concentrated HCl (6.2 M). The final volume was adjusted to 100 ml with water. The concentrated Tris-base buffer was diluted to 10 mM in water for preparation of the MB stock solution.

2.5. Assessment of DNA-strand breaks with the MB

The MB was supplied at a concentration of 37 nmole in each bottle. The MB was dissolved in 372 \( \mu \text{l} \) of 10 mM Tris-base buffer (pH 7.6) to obtain an end concentration of 100 \( \mu \text{M} \) (initial stock concentration). Two (working) concentrations were utilised for the experiments: 0.5 \( \mu \text{M} \) for treatment of the tissue fluid model (a) directly treated with a plasma jet or (b) indirectly treated through a gelatin tissue model, (c) The MB dispersed within gelatin to assess DNA-strand breaks within the tissue model itself, (d) The MB encapsulated within phospholipid vesicles to assess DNA-strand breaks within the cell models.

Figure 1. An overview of the methodology to assess DNA-strand breaks in synthetic models of tissue fluid, tissue and cells. A MB was used to assess DNA-strand breaks. A simplified structure (loop, stem and quencher (black-filled circle), fluorophore (open circle)) of the MB is shown in (a)–(d). Breakage of the MB stem from single- or double-strand DNA breaks results in separation of the quencher/fluorophore leading to a switch-on of fluorescence of the fluorophore (green-filled circle). The MB was dispersed in HEPES to assess DNA-strand breaks in the tissue fluid model (a) directly treated with a plasma jet or (b) indirectly treated through a gelatin tissue model. (c) The MB dispersed within gelatin to assess DNA-strand breaks within the tissue model itself. (d) The MB encapsulated within phospholipid vesicles to assess DNA-strand breaks within the cell models.

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2.6. CF vesicle poration/rupture indicator

A 50 mM concentration of the CF encapsulation buffer was prepared by adding the following to 13 ml water:

- CF—244 mg
- NaCl—7.6 mg
- NaOH—70.2 mg
- HEPES—31 mg
- EDTA—333.7 mg

2.7. Measurement of $\text{H}_2\text{O}_2$ concentration

A mixture of 2 mg ml$^{-1}$ OPD powder and 4 $\mu$g ml$^{-1}$ HRP was dissolved in HEPES. A volume of 200 $\mu$l of the OPD-HRP solution (herein referred to as the $\text{H}_2\text{O}_2$ indicator) was used for the direct treatment of the tissue fluid model and 400 $\mu$l for through-tissue model or through-hydrogel dressing treatment to ensure contact between the solution and tissue model or hydrogel dressing. Treatment with the neutral He gas jet (i.e. with no applied voltage) did not activate the $\text{H}_2\text{O}_2$ indicator (supporting information, figure S1 (stacks.iop.org/JPhysD/50/274001/mmedia)); therefore, all data were normalised to the untreated $\text{H}_2\text{O}_2$ indicator. The $\text{H}_2\text{O}_2$ concentration was determined from a calibration curve constructed from known concentrations of $\text{H}_2\text{O}_2$ solutions prepared in HEPES (supporting information, figure S2).

2.8. Phospholipid vesicle synthetic cell models

Vesicles were composed of 80% DOPC and 20% cholesterol. Although the composition of membranes for human cells is much more complex and variable between cell types and between healthy and diseased cells, phosphatidylcholines...
such as DOPC) are a major lipid found in these cell membranes [83], and cholesterol accounts for approximately 20% of the lipid mass in the cell membrane. A 100 µl stock solution of DOPC/cholesterol was mixed with 200 µl of chloroform, followed by drying with nitrogen gas. Then 2.5 ml of 50 µM of MB or 50 mM CF was added and the mixture was heated to 75 °C for 1 min and allowed to cool to approximately ambient temperature (~25 °C) before being extruded through two 100 nm polycarbonate membranes in a hand-held syringe extruder (Avanti Polar Lipids, model # 610000). Extrusion was repeated 7 times. The extruded solution was transferred through a Sephadex G25 column to remove non-encapsulated dye. Because the column was supplied with a reservoir of anti-bacterial solution, the first step was to flush out the anti-bacterial solution with 5 ml HEPES. This step was repeated twice and the eluate discarded. Then 2 ml of extruded vesicles and 1 ml HEPES was added. This eluate was also discarded. A further 2 ml HEPES was added and the eluate was collected. The eluate was diluted to 25% for vesicles encapsulating the MB and 50% for vesicles encapsulating CF; these concentrations were determined to be optimal from preliminary experiments.

2.9. Gelatin tissue model

A 10% (w/v) gelatin film was prepared by dissolving the gelatin powder in water at 45 °C for 60 min. A volume of 20 ml gelatin solution was poured into an 85 mm diameter (polystyrene) petri dish. The gelatin was allowed to set at 4 °C for at least 12 h before use. The gelatin was cut into approximately 20 × 20 mm² sections; the thickness of the gelatin was 2 mm.

2.10. Hydrogel dressing

‘IntraSite Conformable’ (Smith & Nephew, catalogue # 66000324) hydrogel dressing was utilised for the experiments. ‘IntraSite Conformable’ comprises a hydrogel coating on a non-woven dressing. The hydrogel dressing is supplied folded in half in the packaging. To prepare the dressing, it was unfolded and the half smeared with most of the hydrogel was cut into approximately 20 × 20 mm² sections; the other half was discarded. The dressing was pressed down onto the 96-well plate filled with 400 µl of MB solution or with the H₂O₂ indicator. The side of the dressing coated with most of the hydrogel contacted the solution.

2.11. Microplate reader measurements

A 100 µl volume of the test solutions was transferred to a separate 96-well plate for absorbance or fluorescence measurements. For the H₂O₂ indicator, the absorbance was recorded at 450 nm. Fluorescence of the MB or CF were recorded at an excitation wavelength of 485 nm and emission wavelength of 520 nm.

2.12. Data processing

Preliminary experiments revealed that the neutral He gas jet (i.e. with no applied voltage) alone does not induce DNA-strand breaks in HEPES (supporting information, figure S3). Therefore, the raw fluorescence data were normalised to untreated MB in HEPES according to the following equation:

Normalised fluorescence intensity

\[
= \frac{\text{Fluorescence intensity of test MB suspension}}{\text{Untreated MB suspension}} - 1. \quad (1)
\]

The same equation was also used to normalise the fluorescence data for the CF vesicles to measure membrane poration/rupture:

Normalised fluorescence intensity

\[
= \frac{\text{Fluorescence intensity of test CF vesicles}}{\text{Untreated CF vesicles}} - 1. \quad (2)
\]

All data presented in the column charts represent the mean value from triplicate samples (n = 3) and ± standard error of the mean (SEM).

2.13. Phase-contrast and fluorescence microscopy

Phase-contrast and fluorescence microscopy were performed using an inverted microscope (Nikon, TE-2000) through a 4 × objective. Fluorescence images were captured through a Nikon filter with 455–485 nm excitation and 500–545 nm emission. The same exposure time was used for all samples to enable direct comparison between images. Images were recorded with a Nikon DXM1200C digital camera and processed using NIS-Elements Basic Research v2.2 software.

3. Results

The plasma jet in operation with 0.05 or 0.5 slpm of He is shown in figure 3. With the experimental parameters employed in this study, as seen with the unaided eye, the visible part of the plasma jet was confined within the tube when operated at 0.05 slpm (figure 3(a)), but extended out from the nozzle to a length of 8 mm at 0.5 slpm (figure 3(b)). At 0.05 slpm, the plasma jet did not visibly contact the HEPES target in direct treatment (figure 3(c)) but did contact at 0.5 slpm (figure 3(d)). Using optical emission spectroscopy, we confirmed that the 0.05 slpm plasma jet did not contact HEPES but the 0.5 slpm jet did (data not shown). But in treatments through gelatin, at both flow rates the plasma jets contacted the surface of the gelatin (figures 3(d) and (e)); however, the 0.5 slpm plasma jet exhibited a greater emission intensity and formed a larger contact area with the gelatin target. We take this observation to indicate that the delivery rate of RONS will be higher with the higher flow rate plasma jet than with the lower flow rate plasma jet. In addition, we appreciate that differences in the conductivity of the target might influence the physicochemical properties of the plasma jet upon plasma jet-target interactions [84].

DNA-strand breaks were first assessed for the MB suspended in HEPES using the experimental set-up shown in figure 1(a). The results are shown in figure 4(a). All plasma jet treatments resulted in DNA-strand breaks. We measured H₂O₂ as a surrogate for the total RONS because H₂O₂ is the
major longer-lived RONS generated in aqueous solution for this plasma jet and the experimental configuration utilised in this study [42–44]. Larger treatment times were used with the 0.05 slpm plasma jet in an attempt to obtain similar H2O2 concentrations to those obtained with higher flow rate plasma jet treatments at shorter treatment times.

Figure 4(a) shows that the frequency of DNA-strand breaks increased with plasma jet treatment time. The shortest treatment times of 15 and 30 s with the higher (0.5 slpm) flow rate plasma jet resulted in the lowest frequency of DNA-strand breaks. The longest treatment time of 20 min with the lower (0.05 slpm) flow rate plasma jet resulted in most DNA-strand breaks. The concentration of H2O2 in HEPES after the plasma jet treatments is shown in figure 4(b). The concentration of H2O2 generated with the 0.05 slpm plasma jet was in the range of ~200 µM to 250 µM. Despite much shorter treatment times, the H2O2 concentration range in HEPES after 0.5 slpm plasma jet treatments was higher at >300 µM to ~1 mM. These data show a clear discrepancy in the extent of DNA-strand breaks and the total delivered concentration of RONS. The ability of H2O2 to produce DNA-strand breaks (alone) was tested over the concentration range relevant to these plasma jets; intriguingly, H2O2 (added as a solution to the MB suspension) did not produce any measureable level of DNA-strand breaks (figure 4(c)). This result is mirrored in a previous simulation study by Verlackt et al who showed that the OH• directly damages DNA but H2O2 does not [85]. For the plasma treatment parameters investigated in this study, we did not observe a change in the pH or temperature of HEPES directly treated with the plasma jet.

In the next experiments, DNA-strand breaks were assessed in HEPES below a gelatin target. In the employed experimental set-up, the plasma jet treats the surface of gelatin situated on top of the 96-well, which is filled with a suspension of MB in HEPES (figure 1(b)). Whilst it has been shown in previous studies that plasma jets readily deliver RONS to millimetre depths into tissue models, these targets significantly attenuate RONS delivery, particularly at the onset of plasma treatment [42–45, 47]. Therefore, in this experiment the 0.5 slpm plasma jet was employed for the much longer treatment time of 10 min to increase the H2O2 concentration in the HEPES below the gelatin. As shown in figure 5, no measureable level of DNA-strands breaks was observed in HEPES. This is in contrast to the direct treatment of HEPES, where DNA-strand breaks were readily detected (figure 4(a)) and on a much shorter time-scale. However, it should be noted that the H2O2 concentration delivered by the plasma jet through the gelatin into HEPES was much lower at ~50 µM, when
compared to the direct treatment of HEPES (see figures 4(b) and 5).

The above data implicate other ‘agents’ in the DNA-strand breaks beyond \( \text{H}_2\text{O}_2 \). And moreover, point to the requirement of direct exposure to the plasma jet for DNA-strand breaks to occur. Therefore to investigate this further, DNA-strand breaks were investigated with the MB dispersed directly and uniformly within the gelatin tissue target. The plasma jet was directly applied to the gelatin target surface as shown in figure 1(c). DNA-strand breaks were assessed by fluorescence microscopy of the gelatin. An overlay of one typical phase-contrast and one fluorescence image of the same area are shown in figure 6 to illustrate the correlation between the treatment area on the gelatin surface and fluorescence as a result of DNA-strand breaks in the MB. DNA-strand breaks were not detected in gelatin after 60 s of treatment with the 0.5 slpm plasma jet (data not shown). But, when the treatment was increased to 10 min, DNA-strand breaks were observed at the point of impact of the plasma jet (figure 6(a)). In contrast, no DNA-strand breaks were observed after 10 min treatment with the neutral He gas jet; although the He gas jet did partially dehydrate the gelatin surface (figure 6(b)). Overall, these data show that the plasma jet can also induce DNA-strand breaks in the gelatin tissue model, but this required a treatment time-scale of minutes. Possibly longer treatment times are required to observe DNA-strand breaks in the gelatin tissue model because the shorter-lived and highly-reactive RONS (that are likely to induce significant DNA damage) are extinguished very rapidly in the gelatin matrix (compared to in HEPES). With electron spin resonance spectroscopy, we readily observed the production of the \( \text{OH}^* \) in HEPES, when directly treated with the plasma jet, but not when treated through the gelatin target (supporting information, figure S4). Also, we do not see a further increase in the frequency of DNA-strand breaks after 1 h following plasma jet treatment (data not shown). These data indicate that there is not an appreciable amount of \( \text{H}_2\text{O}_2 \) converted to the \( \text{OH}^* \) over the timescales investigated in this study, and that the DNA-strand breaks observed in this study are likely to be from the \( \text{OH}^* \).

In the treatment of biological cells it is important to consider the phospholipid cell membrane, which is a barrier that exogenous RONS must traverse in order to reach the DNA in the cell interior. Therefore, DNA-strand breaks were next examined with the MB encapsulated within phospholipid vesicles. We appreciate that in real eukaryotic cells, RONS would also need to traverse the nuclear or mitochondrial membrane in order to reach most of the genetic material within the cell. However, for this initial study the MB was packaged into a single vesicle to assess if the RONS that pass through a single phospholipid membrane barrier can react with the DNA within the vesicle interior. The experimental set-up is shown in (figure 1(d)) and the results shown in figure 7(a). The 0.5 slpm plasma jet at the shorter treatment times induced more DNA-strand breaks within the vesicles than longer treatments with the 0.05 slpm plasma jet. (The normalised fluorescence values presented in figures 4(a) and 7(a) cannot be directly compared between the MB experiments, i.e. between the encapsulated MB and free MB (in suspension). This is because (i) different concentrations of MB were used for both experiments and (ii) it was not possible to measure the final concentration of MB encapsulated within the vesicles.)
The integrity of the phospholipid membrane during plasma jet treatment was assessed employing vesicles encapsulating a self-quenched dye (CF). Packed within the vesicles there is no fluorescence and the dye only fluoresces when it is released by membrane poration or vesicle rupture—providing a clear fluorescent switch-on (figure 7(b)) [48, 49, 86]. The results are shown in figure 7(c). The measured level of CF fluorescence was similar for all plasma jet treatments. However, the intensity of CF fluorescence following plasma jet treatments was low at only ~10% of the level obtained with a surfactant (Triton) known to induce complete vesicle lysis. Further, this 10% value is not significant ($p > 0.05$ from a student’s $t$-test), being only marginally above the level obtained with the neutral He gas jet treatments (also shown in figure 7(c)).

For certain medical applications, maximising plasma induced DNA-strand breaks in the target could be advantageous, e.g. in focused cancer therapy. However, in other applications such as the treatment of an open wound, the imperative should be to limit DNA damage. Our data show that it is difficult to inhibit DNA-strand breaks without compromising the RONS concentration delivered by a plasma jet (see figure 4(a)). We venture that the challenge is to reduce or prevent the plasma jet from delivering highly-reactive RONS such as OH$^\cdot$, O$_2^-$, and ONOO$^-$ that readily break DNA. Therefore, in the direct treatment of tissue fluid, one approach to inhibit DNA breaks would be to ‘filter’ the highly-reactive RONS from the plasma jet during treatment. This could potentially be accomplished with the use of a material placed in-between the plasma jet and tissue that enables the (rapid) transport of gases or dissolved RONS from the plasma jet to the tissue. In this scenario, it is anticipated that highly reactive RONS (i.e. those that are more likely to induce DNA-strand breaks) would be extinguished in this material owing to their shorter lifetime and limited diffusion distances. This was tested in the final experiment where a commercial hydrogel dressing (Intrasite Conformable) was placed in-between the plasma jet and HEPES. A photograph during treatment and a diagrammatical representation of the experimental set-up are shown in figures 8(a) and (b) for HEPES treated indirectly with the plasma jet through a hydrogel dressing. Treatment was carried out with the 0.5 slpm plasma jet for 10 min. As shown in figure 8(c), the hydrogel dressing significantly attenuated the level of DNA-strand breaks in HEPES but still enabled the delivery of H$_2$O$_2$ at a relatively high concentration of ~200 $\mu$M; a concentration comparable to HEPES directly treated with the plasma jets (compare figure 8(c) to figure 4(b)). This result adds to the evidence that the more stable, longer-lived RONS from plasma are not responsible for DNA-strand breaks.

4. Discussion

The purpose of this study was to establish a simple methodology to assess DNA damage induced by plasma jets in the context of the use of plasma jets in biology and medicine. Key features of any such methodology are that it should be
and corresponding H$_2$O$_2$ concentration for the same plasma jet of the experimental set-up. (c) Result of DNA-strand breaks through the hydrogel dressing and (b) schematic representation concentration generated in the tissue fluid model underneath the fluid model after plasma jet treatment through a hydrogel treatment time was 10 min.

plates for higher through-put screening with microplate readers. The experiments can also be interfaced with micro-

be utilised to assess DNA-strand breaks from plasma jets in the results presented in this paper show that this methodology can

quickly, especially since DNA-strand breaks in the MB give

rise to an immediate and easily interpretable switch-on of

strand breaks. In addition, the experiments can be performed

conveniently, especially since DNA-strand breaks in the MB give rise to an immediate and easily interpretable switch-on of fluorescence.

Figure 8. Assessment of DNA-strand breaks in the tissue fluid model after plasma jet treatment through a hydrogel dressing (IntraSite Conformable) and the corresponding H$_2$O$_2$ concentration generated in the tissue fluid model underneath the hydrogel dressing. (a) Photograph during plasma jet treatment through the hydrogel dressing and (b) schematic representation of the experimental set-up. (c) Result of DNA-strand breaks and corresponding H$_2$O$_2$ concentration for the same plasma jet treatment. The plasma jet was operated at 0.5 slpm He and the treatment time was 10 min.

reproducible, cost and time effective, not require highly-specific expertise or analytical equipment, and that the data yielded should be straightforward to interpret. As seen in the graphs, the SEM of the replicate data is relatively small indicating good experiment reproducibly. We attribute the good level of reproducibility to little batch-to-batch variation in the synthetic models because the fabrication processes are straightforward, and because experimentation with synthetic tissue models avoids biological variability. Generally, the costs of the reagents are low: the reagents to make the tissue fluid (HEPES), tissue model (gelatin) and cell models (phospholipid vesicles) are not expensive; the major cost is the MB, but the MB is used at a low concentration in 96-well formats to reduce volume requirements. The cost of the equipment is also relatively low and most of the equipment is standard to chemical and biology laboratories; such as the small handheld extruder used for making vesicles, and the microplate reader and fluorescence microscope for measuring DNA-strand breaks. In addition, the experiments can be performed quickly, especially since DNA-strand breaks in the MB give rise to an immediate and easily interpretable switch-on of fluorescence.

The next issue is the versatility of the methodology. The results presented in this paper show that this methodology can be utilised to assess DNA-strand breaks from plasma jets in the tissue fluid model, within the tissue model, and within the cell models. The experiments can also be interfaced with microplates for higher through-put screening with microplate readers.

It is important to also critically assess the value of the methodology: can useful data be derived and is it relevant to real biological in vitro and in vivo experiments? Therefore, the remaining discussion will focus on the significance of the initial results with respect to the research field of ‘plasma medicine’, as well as limitations of the methodology and scope for further improvements.

The major result was that plasma jets have the potential to damage DNA in tissue fluid, tissue and cell models. This is not surprising because as discussed earlier, plasma jets generate highly-reactive RONS that have previously been shown to break DNA-strands [78–82]. In the treatment of life-threatening indications such as cancers and chronic wounds, it would be advantageous to target the destruction of cancerous cells within tumours or bacterial cells within biofilms using plasma jets to damage DNA in these cell types. For life-threatening indications, any risk associated with respect to inadvertent DNA damage in healthy cells (in the surrounding tissue) is perhaps reasonably low considering that not all DNA aberrations cause disease and the cellular machinery of healthy cells is well equipped to repair most damaged DNA [87, 88]. On the other hand, the data in this study suggest that given there are always DNA-strand breaks (in direct application of plasma to the tissue fluid model) there remains some level of potential risk, and in such a case the risk ought to be factored in when considering non-critical clinical applications of plasma jets such as for cosmetics (e.g. wrinkle treatments and teeth whitening).

Targeted DNA damage with plasma jets could be the objective in the treatment of cancer. Often elevated levels of DNA damage are linked to an increase in frequency of cancer cell death induced by plasma jets in vitro [35, 89]. This is a similar approach to that adopted with ionising radiation therapy in that damaged DNA in the cell blocks further cell proliferation; and the key is to target the radiation to the diseased tissue only [90]. However, despite ongoing advances in techniques utilised for ionising radiation therapy, 95% of patients who receive ionising radiation therapy consequently suffer from skin damage [91]. In this respect plasma has an advantage over ionising radiation in that it generally does not damage skin. Another potential advantage is that plasma jets can be highly targeted to treat cancer cells [92] with the miniaturisation of the plasma jet nozzle down to nanometre dimensions [93]. In addition to the plasma jet induced breakage of DNA-strands in HEPES (tissue fluid model), the plasma jet also induced DNA-strand breaks in gelatin (tissue model). This demonstrates that it might be possible to use plasma jets to damage DNA in a solid tissue mass such as a tumour. Importantly, the plasma jet is unlikely to cause DNA damage far underneath a tumour because no DNA damage was observed in HEPES underneath the gelatin target. Overall, these data show that plasma jets could potentially be utilised in the future to damage DNA at specific sites within tissue for targeted cancer therapy.

Another potentially interesting observation is that the lower flow rate (0.05 slpm) plasma jet induced more DNA-strand breaks compared to the higher flow rate (0.5 slpm) plasma jet when the MB was suspended in HEPES; whereas the opposite trend was observed when the MB was encapsulated within...
vesicles. We propose three not mutually exclusive explanations for the opposing trends. We start from the premise that H$_2$O$_2$ does not induce DNA-strand breaks. Therefore, we suspect that it is short-lived, highly-reactive RONS that are responsible for the observed DNA-strand breaks. Treatment of MBs in HEPES with the lower flow rate, but substantially longer treatment times, results in greater damage of the MB than at the higher flow rates (at much reduced treatment times) because the total number of interactions between the short-lived, highly-reactive RONS and MBs is greater in the former case. But when the MB is encapsulated, these highly-reactive RONS most probably react almost entirely with the vesicle’s phospholipid membrane before reaching the DNA within. Therefore, we propose the reversed result arises because:

1. The higher H$_2$O$_2$ concentration generated by the higher flow rate plasma jet becomes important as it facilitates the oxidation [94, 95] and permeabilisation of the phospholipid membrane, which enhanced the ingress of any remaining highly-reactive RONS [55];

2. The higher flow rate plasma jet extends the plasma jet to contact the liquid, which should increase the electric field around the vesicles. The maximum electric field generated by the plasma jet in this study is 2.5 kV cm$^{-1}$. Although not investigated in this study, we know similar plasma jets generate small packets of charge (usually referred to as ‘plasma bullets’) that carry a net charge in the order of $10^{-9}$ C with estimated particle densities between $10^{0}-10^{11}$ cm$^{-3}$ that can propagate to distances of several tens of millimetres from the plasma jet nozzle [96]. This phenomenon is usually described as an ‘ionisation front’ and has been termed a ‘guided streamer’ because its propagation is confined to within the He gas flow exiting the plasma jet nozzle [97, 98]. The magnitude of the peak streamer head electric field has been reported to be up to ~5–7 kV cm$^{-1}$ [97]. A further increase in electric field from the ‘plasma bullets’ contacting the HEPES might have transiently increased the permeability of the phospholipid membrane during plasma jet treatment. This would enhance the ingress of the highly-reactive RONS. Previously, in a computational simulation study, Yusupov et al has shown that the synergistic action of electric field with H$_2$O$_2$ potentially increases phospholipid membrane permeability that increases the ingress of RONS into cells [54]. This perhaps occurs on a very fast time-scale with only small nanometre diameter pore openings in the phospholipid membrane [99]. This would explain only minimal leakage of the encapsulated CF from the vesicles with plasma jet treatment.

3. The gas flow itself imparts greater mechanical forces onto the phospholipid membrane of the vesicles [100]. Consequently, greater mechanical forces exerted onto the phospholipid membrane by the higher flow rate plasma jet, enhanced the ingress of RONS.

But we can reasonably speculate based on the present data and results from our previous studies. With respect to DNA damage, any direct DNA-strand breaks induced by the plasma jet would be permanent, but further DNA strand breaks would not be expected to occur in the longer term as the highly-reactive RONS would be extinguished rapidly after completion of the plasma jet treatment. In a ‘real’ cell, the cellular machinery can repair damaged DNA [87, 88]. However, further DNA aberrations could result in the longer term if cell function such as the cell cycle is altered by the plasma jet treatment. With respect to modifications of the phospholipid membrane, our data to date indicate that the increased permeability of the phospholipid membrane vesicles to RONS after plasma jet treatment is permanent. This enables the ingress of longer-lived RONS many hours after completion of the plasma jet treatment [48, 49]. However, it is difficult to ascertain if the longer term increased permeability of the phospholipid membrane to RONS is attributed to the initial plasma jet treatment or the fact that longer-lived RONS (e.g. H$_2$O$_2$) remain in the solution for many hours after the plasma jet treatment. Once again, in a ‘real’ cell the effects are likely to be different. Through evolution cells have developed mechanisms for repairing membranes [101].

In certain circumstances, such as stimulation of wound healing or tissue regeneration, it could be advantageous to only deliver RONS that stimulate cell activity (e.g. proliferation and migration). In this study, we have seen that a hydrogel dressing placed in-between the plasma jet and HEPES or gelatin targets can significantly attenuate DNA-strand breaks but still enable the transport of H$_2$O$_2$ (and by inference other longer-lived RONS) to the targets. Delivery of controlled doses of H$_2$O$_2$ was previously shown to have beneficial effects on wound healing in vivo [102]. Plasma jet treatment through a hydrogel dressing is a simple method to potentially stimulate wound healing with the added advantage of not needing to remove the dressing for treatment.

Having considered the advantages, it is worth considering the limitations of the methodology we report, which we discuss with a view to future improvements. The major limitations are that the tissue fluid, tissue and cell models are without real biological material, antioxidants and living cells, and the system is static with no physiological fluid flow. Therefore, the level of DNA-strand breaks observed in this study could be considered as a worst case scenario. The physicochemical properties and inherent antioxidant defences of real biological tissue fluid and tissue would to some extent mitigate the transport of the RONS likely to induce DNA-strand breaks. Additional features that could be in-built to models include the incorporation of proteins, enzymes and antioxidants and cross-linking agents. These could be incorporated into either the tissue fluid or tissue models to mimic different physiological environments. In addition, it is possible to incorporate living cells into, for example, a mimic of a 3D tumour [103, 104]. This would enable us to study cellular signalling events within a solid tumour; intercellular signalling might also facilitate the plasma-generated signal to reach cells deeper within the tumour, which could potentially amplify DNA damage. It should also be possible to incorporate fluidic systems with
small pumping systems to mimic physiological flow. These features could be incorporated into the future design of the synthetic models to help improve our understanding of plasma interactions with biological systems.

5. Conclusions and future directions

A methodology is established to assess DNA-strand breaks induced by a He plasma jet in synthetic models of tissue fluid, tissue and cells. The plasma jet induced DNA-strand breaks in the tissue fluid and tissue models. DNA-strand breaks were also detected in the cell model without significant poration or rupture of the phospholipid membrane. DNA-strand breaks were significantly reduced in the tissue fluid when a hydrogel dressing was situated in-between the plasma jet and tissue fluid during treatment, but H2O2 (and potentially other RONS not measured) could still be transported through the dressing. The synthetic models could be improved in the future to mimic more realistic biological systems. But major advantages of the methodology are its cost effectiveness and it affords rapid analysis of DNA-strand breaks in different pseudo biological environments. Developing a more detailed understanding of plasma-DNA interactions in different biological environments should help the future development of plasma medical therapies.

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