



Citation for published version:

Hong, S-H, Szili, EJ, Jenkins, ATA & Short, RD 2014, 'Ionized gas (plasma) delivery of reactive oxygen species (ROS) into artificial cells', *Journal of Physics D: Applied Physics*, vol. 47, no. 36, 362001.
<https://doi.org/10.1088/0022-3727/47/36/362001>

DOI:

[10.1088/0022-3727/47/36/362001](https://doi.org/10.1088/0022-3727/47/36/362001)

Publication date:

2014

Document Version

Peer reviewed version

[Link to publication](#)

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Ionized Gas (Plasma) Delivery of Reactive Oxygen Species (ROS) into Artificial Cells

Sung-Ha Hong,¹ Endre J. Szili,^{2,} A. Toby A. Jenkins,¹ and Robert D. Short^{2,*}*

¹Department of Chemistry, University of Bath
Bath, BA2 7AY, UK

²Mawson Institute, University of South Australia, Adelaide, SA 5095 (Australia)

Corresponding authors: endre.szili@unisa.edu.au and rob.short@unisa.edu.au

Hong and Szili are co-first authors

This study was designed to enhance our understanding of how reactive oxygen species (ROS), generated ex-situ by ionized gas (plasma), can affect the regulation of signalling processes within cells. A model system, comprising of a suspension of phospholipid vesicles (cell mimics) encapsulating a ROS reporter, was developed to study the plasma delivery of ROS into cells. For the first time it was shown that plasma unequivocally delivers ROS into cells over a sustained period and without compromising cell membrane integrity. An important consideration in cell and biological assays is the presence of serum, which significantly reduced the transfer efficiency of ROS into the vesicles. These results are key to understanding how plasma treatments can be tailored for specific medical or biotechnology applications. Further, the phospholipid vesicle ROS reporter system may find use in other studies involving the application of free radicals in biology and medicine.

The direct application of plasma (ionized gas) to living tissue has potential in wound care [1], cancer treatment [2], disinfection [3], dentistry [4] and regenerative medicine [5]. Encouraging results over the past decade has included successful randomized clinical trials [6, 7]. These exciting results provide a solid basis for claims of a new medicinal technology, termed 'plasma medicine' [8].

Inert gas plasmas impinging on, or mixed with air, are rich sources of reactive oxygen and nitrogen species (RONS) [8]. Perhaps, as a consequence of this, the efficacious effects of plasmas (when applied to tissue) are often linked to the RONS generated or delivered by the plasma to the biological target. Many of the RONS produced by plasma are also produced *in vivo*; within inter- and intra- cellular environments and are known to regulate key biochemical pathways, inducing chemical and physical changes in cells [9-13]. In a recent review, Graves summarised how plasma-generated RONS might activate / deactivate a number of well-established biological processes [13].

Experiments have been devised to demonstrate the role of plasma generated RONS in cell proliferation, migration and angiogenesis [14-17] and to provide insight into the mechanisms involved in the plasma deactivation of bacteria [18-20] and tumours [2, 21, 22]. Although plausible hypotheses for the successful application of plasma to medical treatment are being advocated, *there remains a major chasm between the plasma-induced 'biological outcomes' and a fundamental, underpinning knowledge of how plasma-generated RONS interact with cells and tissue.*

In order to address this issue, we developed a 'tissue model' to study the plasma interactions with phospholipid vesicles (mimics of cell membranes) encapsulated within a gelatin matrix (surrogate of tissue) [23]. We discovered that plasma can rupture vesicles > 150 μm below the gelatin surface. Recently, we showed that plasma delivers ROS from 150 μm to 1.5 mm into gelatin [24]. However, we could not unambiguously claim that plasma-derived reactive oxygen species (ROS) or reactive nitrogen species (RNS) can interact with cells in a hydrated biological environment.

In this study, we focus on the plasma jet delivery of ROS into phospholipid vesicles (i.e. artificial cells) suspended in 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) solution (pH 7.4). Specifically, we compared the plasma delivery of ROS into vesicles to treatments with hydrogen peroxide (H_2O_2). H_2O_2 is a signalling molecule produced in many (possibly all) tissues *in vivo* [10]. When used as an exogenous ROS source, H_2O_2 can stimulate cell division at low concentrations (1 μM) [25], but is cytotoxic at slightly higher concentrations (> 10 μM) [10]. Biomedical plasma sources produce H_2O_2 in abundance in biological media from low (few μM range) [26] to high (mM range) [27] concentrations.

Vesicles (of 100 nm diameter) encapsulating 2,7-dichlorodihydrofluorescein (DCFH), a chemical indicator for a broad range of ROS [10], were used to monitor the plasma delivery of ROS into the vesicles in HEPES buffer at pH 7.4. Non-fluorescent DCFH is converted by

ROS to the highly fluorescent 2,7-dichlorofluorescein (DCF) product [28, 29]. To investigate vesicle rupture, 50 mM of 5,6-carboxyfluorescein (CF) was encapsulated into 100 nm diameter vesicles. At this concentration, the dye is quenched inside the vesicles. The dye is released when the integrity of the membrane is compromised (*e.g.* from vesicle rupture or perforation), resulting in an increase in the fluorescence signal of CF. The artificial cell models are shown in scheme 1. Analysis of ROS and vesicle rupture were performed in two separate experiments because of the overlapping excitation / emission wavelengths for the dyes. Vesicles were treated with a plasma jet where helium gas is purged through a glass tube; the plasma is then ignited by supplying a high voltage potential across two external hollow electrodes surrounding the tube and the gas flow launches the plasma into the atmosphere (figure 1). The helium plasma jet was operated under conditions that produced a plasma plume of 5 mm in length into the ambient air (figure 1). Treatment was carried out using a relatively low flow rate of 100 ml / min and at a distance of 15 mm from the top of the test well to the end of the glass tube. Under these conditions, the plasma did not contact, and the gas flow did not disrupt, the surface of the test solution (as seen by the naked eye). We refer the reader to SI-1 (Supporting information) for a detailed description of the experimental methods.

Figure 2a shows that the CF fluorescence intensity of the vesicle suspension did not increase immediately after plasma jet treatment for plasma jet treatment times (t) of 1, 5 and 10 min. Because the plasma jet treatment does not quench the fluorescence intensity of CF (*i.e.* no change in fluorescence was detected for unencapsulated CF in HEPES exposed to plasma, SI-2 Supporting information), we can conclude that the CF dye was not released into the HEPES, and therefore the vesicle membranes were not lysed by the plasma jet treatment. On the other hand, the fluorescence intensity of the vesicles encapsulating DCFH increased as function of the plasma jet treatment time (*i.e.* dosage). Therefore, predominantly the plasma jet delivers ROS into the vesicles without damaging the vesicle membranes. The concentration of DCFH encapsulated within the vesicles was not a limiting factor in these experiments; in SI-3 (Supporting information) we show a further capacity of the dye to oxidise after two 10 min plasma jet treatments. Treatment with 1% (v/v) Triton™ X-100 (surfactant that disrupts vesicles) confirmed the CF dye is released upon vesicle lysis and further, that rupture of the vesicles does not increase fluorescence of the DCFH ROS reporter (figure 2b). Treatment with the neutral helium gas (*i.e.* plasma off) had no effect on vesicle lysis or delivery of ROS (figure 2b). In addition to 100 nm-sized vesicles, the plasma jet readily delivered ROS into giant unilamellar vesicles (GUVs) of several μm in diameter (approaching the dimension of a cell) without notable damage to the vesicle membrane (SI-4 Supporting information).

The next experiment was designed to study the effects of the longer-lived plasma ROS, without direct exposure to the plasma beam and to VUV / UV radiation (produced in the plasma jet); removal of the plasma beam removes very short-lived radicals. It is known that VUV / UV can induce chemical and physical changes in cells [8]. Diffusion of the plasma

generated longer-lived ROS was compared to a relatively stable reactive species known to be generated by plasma (*i.e.* H₂O₂). HEPES (with no vesicles) was treated with the plasma jet and the plasma-activated liquid (PAL) was mixed with DCFH-containing vesicles. Approximately 56, 74 and 105 μM of H₂O₂ was produced by the plasma jet treatment in HEPES for $t = 1, 5$ and 10 min, respectively (SI-5 Supporting information). Based upon the fluorescence intensity of oxidised DCFH, we deduce that a higher quantity of ROS was delivered inside the vesicles from the PAL in comparison to the corresponding concentrations of H₂O₂ solutions (figure 3a-b). This result indicates that the PAL retains a heterogeneous mixture of longer-lived ROS that can participate in cell oxidation. These data were acquired immediately after plasma jet treatment (defined as 0 h). Even after 15 h of incubation, ROS in the PAL still diffused into the vesicles (figure 3a). This result indicates that plasma-generated ROS may *in situ* have appreciable half-lives such that plasma generated ROS could even oxidize new cells (generated from cell division) many hours after the initial plasma treatment.

Although the above data show that a plasma jet generates ROS that interact with vesicles over a sustained period (without damage to the membrane), the *in vivo* situation could be somewhat different: the highly proteinaceous environment of the extracellular matrix may affect the delivery of ROS into cells. *E.g.* Tian *et al* showed through mathematical modelling, that plasma-derived ROS are largely ‘consumed’ by alkane hydrocarbons [30]. Furthermore, the serum of cell culture media contains amino acids that readily react with the plasma reactive species [31] and antioxidant enzymes such as catalase that protect cells against oxidation [32].

We tested this by performing the plasma jet treatment on DCFH-containing vesicles in HEPES supplemented with two different concentrations of fetal bovine serum (FBS), which is a common additive in cell culture media. The level of DCFH oxidation within the vesicles was significantly reduced in HEPES containing 10% (v/v) FBS (figure 4). FBS is commonly used at 10% (v/v) for *in vitro* cell culture. Delivery of ROS into the vesicles was almost completely prevented in HEPES supplemented with a higher concentration of FBS at 50% (v/v). The plasma jet treatment did not lyse vesicles containing CF in HEPES supplemented with 10% or 50% (v/v) FBS (SI-6 Supporting information). Therefore, the reduced amount of ROS delivered by the plasma jet into the vesicles can be attributed to the reaction of ROS with the serum ingredients and / or from anti-oxidizing enzyme activity.

Quantification of the ROS delivered by the plasma jet into cells is necessary to obtain a firm link between the role of plasma generated ROS and cellular signalling processes. We adapted the formula from Loetchutinat *et al* to measure the plasma jet delivery of ROS into vesicles [33]:

$$\frac{d[\text{DCF}]}{dt} = k_{\text{encap}}[\text{DCFH}][\text{ROS}_i]$$

Here the rate of DCFH oxidation to DCF is $d[\text{DCF}]/dt$; the second order rate constant for oxidation of DCFH (with H_2O_2) is k_{encap} (where *encap* refers to DCFH encapsulated within vesicles); the DCFH concentration is $[\text{DCFH}]$ and the concentration of ROS inside the vesicles is $[\text{ROS}_i]$. The $[\text{ROS}_i]$ was determined experimentally as detailed in SI-7 (Supporting information). The $[\text{ROS}_i]$ delivered by the plasma jet into the entire vesicle suspension seemed low (*e.g.* 3.32×10^{-8} M at 1 h after 1 min of plasma jet treatment, Table 1). However, this value converts to 0.633×10^{-4} M μm^{-3} when adjusted to moles / unit volume (Table 1). This is orders of magnitude higher than steady-state levels of ROS in cells at $\sim 1.26 \times 10^{-12}$ M μm^{-3} (assuming a 10 μm diameter spherical cell) [33]. In comparison, a lower $[\text{ROS}_i]$ was measured in vesicles treated with PAL or H_2O_2 ; although these values are still well above steady-state ROS levels in cells (Table 1). Because a significant amount of plasma generated ROS can be delivered into cells, it is possible that plasma generated ROS may regulate cellular processes through the oxidation / modification of the internal cell components as opposed to more indirect mechanisms involving the modification of extracellular matrix biomolecules or the external cell membrane / receptors that in turn, leads to the up-regulation of ROS within the cell. We acknowledge that the cellular machinery is equipped to regulate ROS levels; however, we also note that it is energetically more favourable for cells to repair or replace damaged biomolecules rather than maintaining higher levels of antioxidant defences against ROS [10].

The long term goal (and major impact) is the use of plasma to synthetically generate RONS that intervene in known biological processes associated with disease or tissue regeneration [13]. This goal is based upon the capability to develop new medical therapies whereby plasma generated RONS are directly delivered to sites of disease or injury. This capability has the potential to exceed what can be achieved using conventional drug therapies. However, understanding the fundamental role of RONS and free radicals in biology and medicine is confounded by the complexity of the biological environment and intracellular signalling pathways and the difficulty to accurately detect and quantify RONS in the cell. Other effects, such as oxidative artefacts in cell culture media, can lead to misinterpretation of results [32]. Therefore, the ability to analyse the interactions of plasma generated RONS in model systems under controlled and repeatable conditions should significantly help in expediting our fundamental understanding of the role of plasma in medicine. This study shows that plasma delivers ROS into cells above steady-state levels in cells (as reported in the literature) and for a sustained period (at least 15 h). These results might be linked to the observed biological effects of plasma generated RONS such as plasma-induced wound healing, stem cell differentiation and cancer cell destruction. The phospholipid vesicle ROS reporter system may also find use in a range of other studies involving free radicals in biology and medicine.

Acknowledgements

The authors thank the Wound Management Innovation CRC for partially funding this work through project RP 2.11. The authors thank Bacteriosafe for providing the travel award that

enabled us to carry out this research project, and EC-FP7 funding of programme 245500 Bacteriosafe, EC grant: KOALA 295155.

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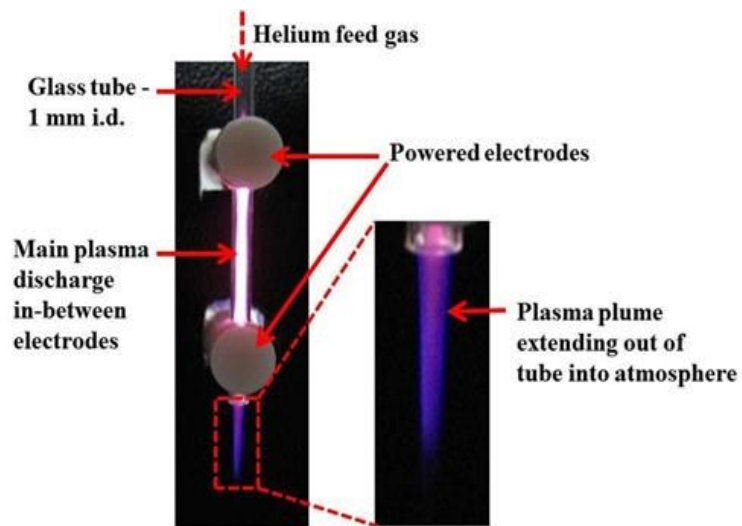


Figure 1. Photograph of the helium plasma jet in operation.

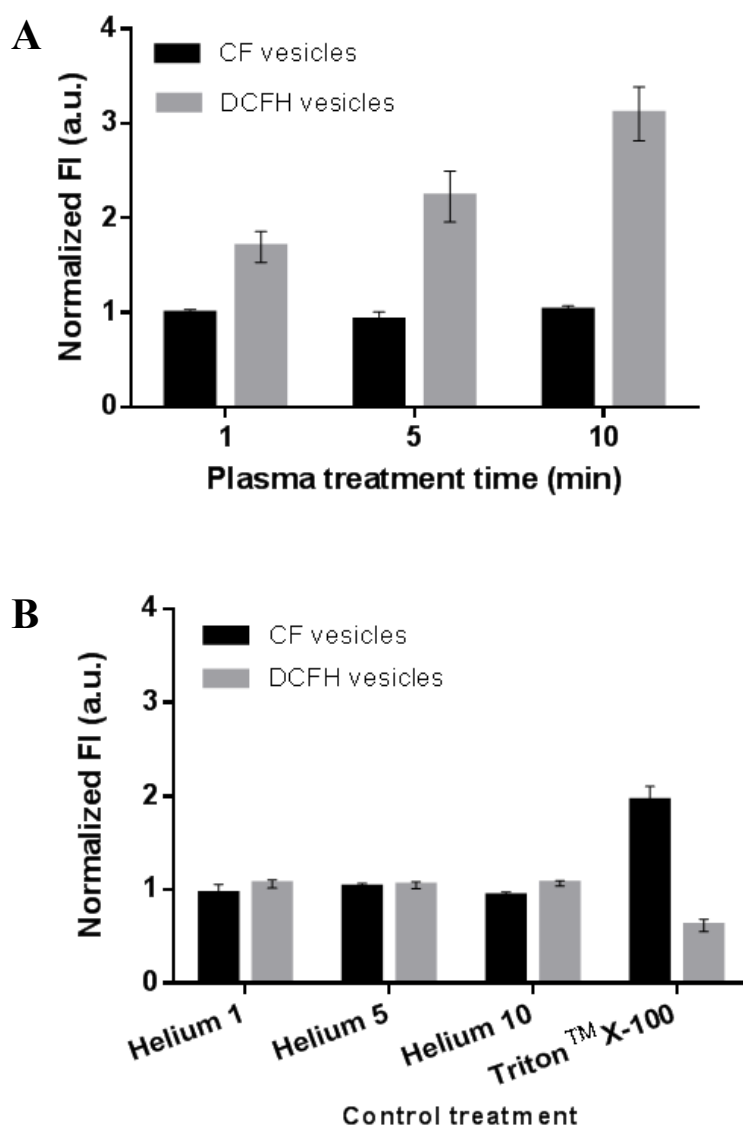


Figure 2. Plasma jet delivers ROS non-destructively into phospholipid vesicles. (A) Normalized fluorescence intensity (FI) of 100 nm diameter vesicles encapsulating CF or DCFH after plasma jet treatment. (B) Normalized FI of vesicles encapsulating CF or DCFH after control treatments with Triton™ X-100 (positive control for vesicle lysis) and the neutral helium (*i.e.* plasma off). Helium 1, 5 and 10 in (B) refers to the helium treatment time.

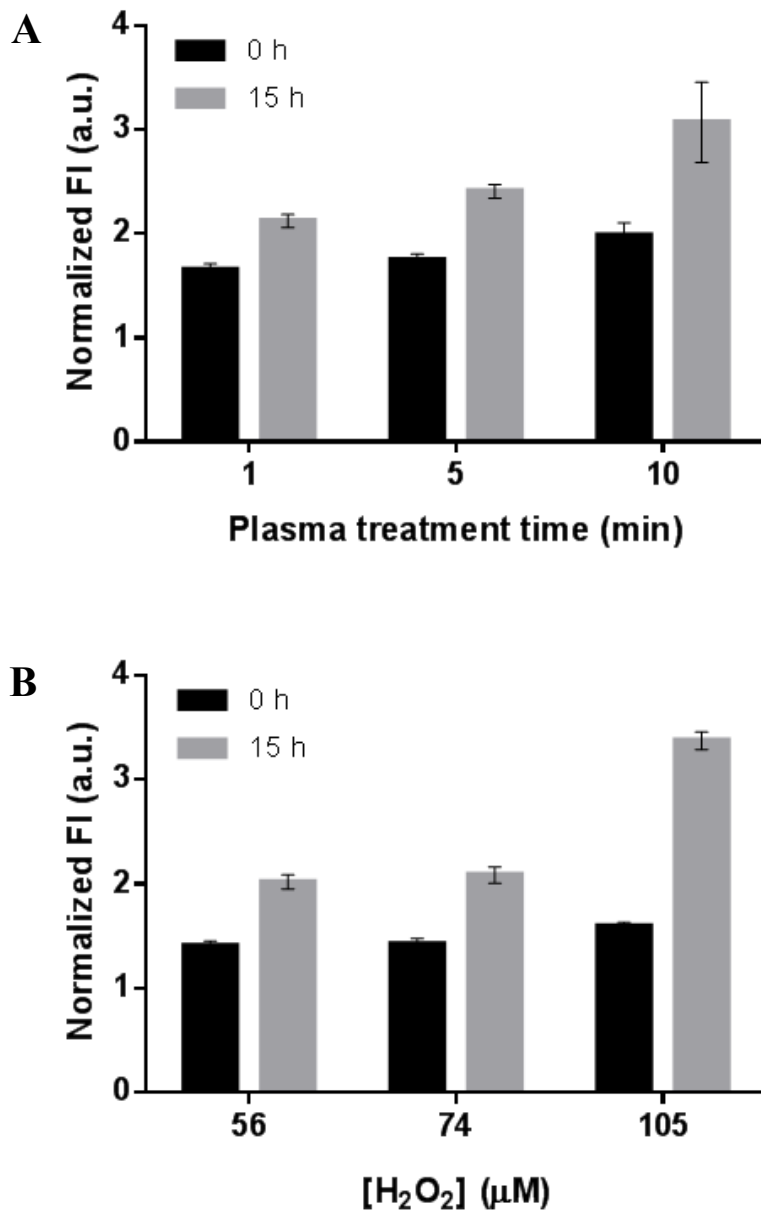


Figure 3. Plasma generated ROS are long-lived and penetrate phospholipid vesicles over a sustained 15 h period. (A) Normalized fluorescence intensity (FI) of 100 nm diameter vesicles encapsulating DCFH after addition of the PAL. (B) Normalized FI of 100 nm vesicles encapsulating DCFH after addition of the H_2O_2 solutions. The H_2O_2 concentrations are matched to those measured in PAL; *i.e.* plasma jet treatment t of 1 = 56 μM , 5 = 74 μM and 10 = 105 μM .

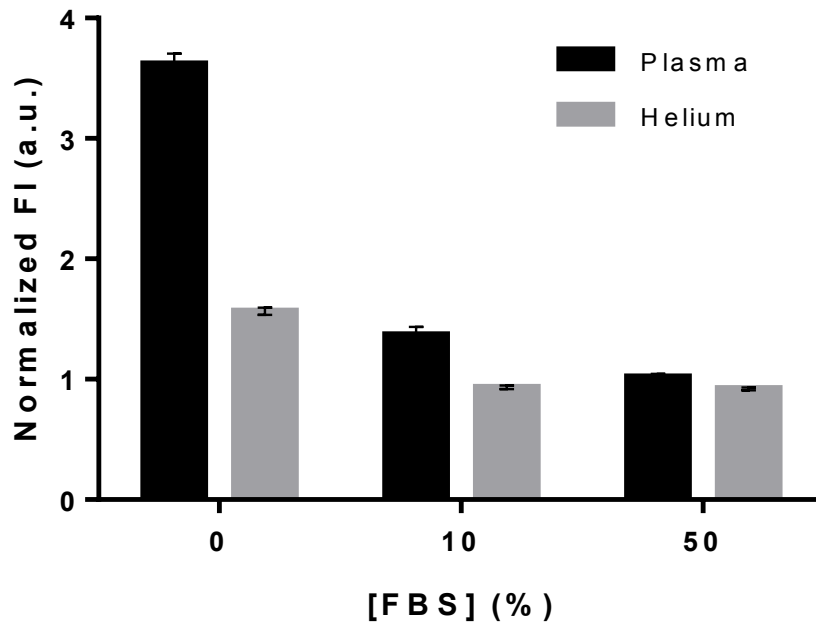
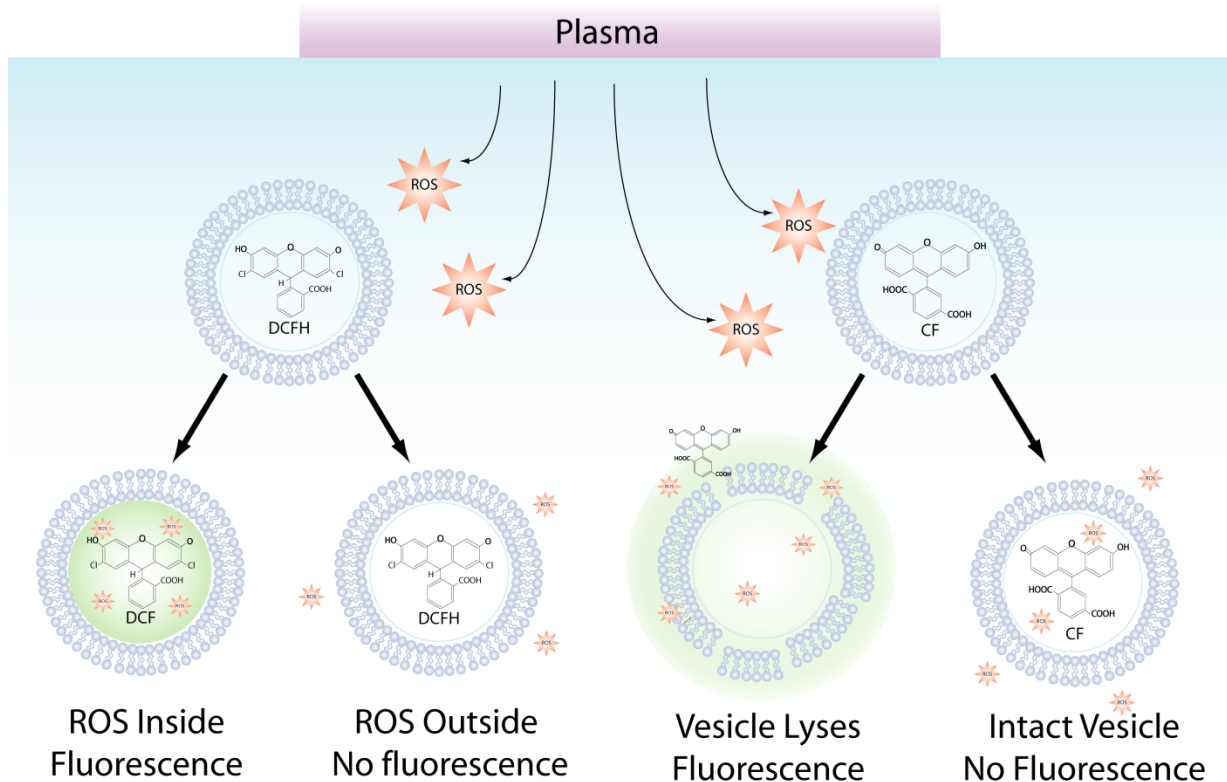


Figure 4. Efficiency of the plasma delivery of ROS into vesicles is significantly reduced in the presence of cell culture serum. Vesicles encapsulating DCFH in HEPES were treated with the plasma jet in the presence of 0%, 10% and 50% FBS. Plasma jet treatment was compared to treatment with the neutral helium (*i.e.* plasma off).



Scheme 1. Diagrammatical representation of the artificial cell models (vesicles) used to study the plasma delivery of ROS into vesicles and plasma induced vesicle damage. Vesicles encapsulating 2,7-dichlorodihydrofluorescein (DCFH) were used to monitor the plasma delivery of ROS into vesicles, as shown on the left. Vesicles encapsulating 5,6-carboxyfluorescein (CF) were used to assess if plasma treatment induced vesicle lysis, as shown on the right.

Table 1. Concentration of ROS delivered into the vesicles by the plasma jet, from the plasma-activated liquid (PAL) and from the H₂O₂ solution. The plasma jet readily delivers ROS into vesicles at concentrations well above steady-state levels observed in cells.^[a]

Treatment condition	[ROSi] @ 1h (x10 ⁻⁸ M)	[ROSi] @ 1h per volume (x10 ⁻⁴ Mμm ⁻³)	[ROSi] @ 15h (x10 ⁻⁸ M)	[ROSi] @ 15h per volume (x10 ⁻⁴ Mμm ⁻³)
Plasma Jet 1	3.32 ± 0.485	0.633 ± 0.096	4.13 ± 0.349	0.788 ± 0.067
Plasma Jet 5	16.5 ± 0.643	3.14 ± 0.123	19.2 ± 1.67	3.66 ± 0.318
Plasma Jet 10	31.8 ± 2.40	6.07 ± 0.458	34.7 ± 0.320	6.63 ± 0.061
PAL 1	4.29 ± 0.250	0.819 ± 0.058	5.22 ± 0.024	0.996 ± 0.005
PAL 5	7.34 ± 0.361	1.40 ± 0.069	7.33 ± 0.098	1.40 ± 0.019
PAL 10	8.62 ± 0.250	1.65 ± 0.048	8.88 ± 0.083	1.70 ± 0.016
56 μM H ₂ O ₂	1.99 ± 0.043	0.380 ± 0.008	2.09 ± 0.005	0.399 ± 0.001
74 μM H ₂ O ₂	2.09 ± 0.581	0.399 ± 0.011	2.16 ± 0.004	0.413 ± 0.001
105 μM H ₂ O ₂	2.43 ± 0.276	0.464 ± 0.005	2.38 ± 0.001	0.455 ± 0.005

^[a]Steady-state levels of ROS calculated in cells from values provided by Loetchutinat *et al* [33] and assuming that the cell is spherical with a diameter of 10 μm.