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New insights on molecular internalization and drug delivery following plasma jet exposures



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ABSTRACT

In this study, we evaluated cold atmospheric plasmas as a physical drug delivery tool for human cervical cancer HeLa cells and murine breast carcinoma 4T1 cells. Different cell exposure protocols – plasma jet, plasma treated medium, and combinations of plasma-induced electric field and plasma treated medium- have been proposed and assessed to provide new insight on plasma-induced uptake mechanism. Cell culture medium composition and volume are key parameters to achieve an efficient molecular uptake. The plasma device enabled the delivery of molecules having 150 kDa-size into 4T1 cells. For the first time to our knowledge, substance uptake kinetics after plasma treatment were investigated. The percentage of positive cells for propidium iodide and an anti-cancer agent, doxorubicin, was higher when the drugs were added a few minutes after treatment. The Plasma treated medium was not found to be as efficient as direct plasma treatment in 4T1 cells while allowing an efficient delivery in HeLa cells. Uptake levels as high as $39.3 \pm 2.9\%$ and $40.1 \pm 9.5\%$ for HeLa and 4T1 cells respectively were achieved for optimized operating conditions, for which the viability of the cells was not severely affected. We also observed that plasma treatment induced the formation of actin stress fibers into cells revealing a mechanical stress.

1. Introduction

The development of efficient and safe drug delivery methods is still highly challenging. Techniques known as electroporation and sonoporation are based on the use of physical vectors such as electric pulses or pulsed ultrasound combined with microbubbles. These techniques have been widely used *in vitro* and *in vivo* for drug (Gibot et al., 2013; Kotopoulos et al., 2014) or gene delivery (Manta et al., 2017; Mir, 2014) for more than two decades (Gelker et al., 2019). Electroporation methods are based on the use of electric fields and were first discovered by Neumann et al. in 1982 (Neumann et al., 1982). They found that short-time electric impulses of a few microseconds increased the uptake of DNA into mouse lymphoma cells. Their hypotheses were based on the enhancement of cross membrane transport through the stabilization of permeation sites and a pore configuration process. Electroporation enabled the entry of normally impermeable macromolecules into cells. It has even been used in human trials for gene delivery for the treatment of tumors. Efficient uptake of molecules and DNA by such methods depends on specific parameters, electroporation system design, electrical features, target cells and tissues, and the molecules of interest.

The versatility of these techniques allows for multiple types of tissues and cells accounting for different combinations of set electrical parameters. During the past few years, a novel technology using ionized gas, so called plasma, has been described. Cold atmospheric-pressure plasmas have shown to be interesting tools to treat different biological samples. A wide range of applications of cold atmospheric-pressure plasmas have been investigated including sterilization and decontamination (Klämpfl et al., 2012), wound healing (Schmidt et al., 2017), tumor treatment (Vandamme et al., 2010; Dubuc et al., 2018), tooth bleaching (Sun et al., 2010), dermatology (Heinlin et al., 2010; Kalghatgi et al., 2015; Daeschlein et al., 2018) and anti-aging in cosmetics (Heinlin et al., 2010; Daeschlein et al., 2018; Valnattajomran and Busco, 2019; von Woedtke, 2019). Plasma is an ionized gas with several components including excited species, photons (including UVs), and charged particles. Beside those species, there is also an electric field generated in the environment of the plasma. Cold atmospheric plasmas are generated by ionizing gas with an electric discharge and may be used to treat biological targets. Cold plasmas delivered in ambient air are characterized by the generation of an electric field and the production of reactive oxygen and nitrogen species, so-called RONS

(Graves, 2014). While thoroughly studied, many aspects of how plasma interacts with biological samples is still far from being understood. Applications in cell membrane uptake using cold plasma have also been studied (Leduc et al., 2009). According to the literature, the use of plasma can also allow gene transfer (high-weight molecular uptake). Ogawa et al. first initiated this technique in 2005 by transfecting neuronal cells with plasmid DNA encoding GFP by plasma treatment (Ogawa et al., 2005). Later on, Sakai et al. also used a plasma generator for transfection and the flow cytometry to measure transfection efficiency (Sakai et al., 2006). Mechanisms involved in plasma-induced transfection seemed to be similar to electroporation. Kaneko et al. induced fibroblasts to internalize YOYO-1, a DNA intercalating fluorescent dye inside the nucleus (Kaneko et al., 2015), with helium plasma treatment. They observed a local cell detachment with high uptake on the border and the middle of the treated area. In a previous study (Vijayarangan et al., 2017), using a pulsed plasma jet, the plasma parameters involved in molecular uptake of HeLa cells monolayer were defined. Upon plasma jet treatment, a circular impact zone with internalized probes was evidenced on HeLa cells monolayer. Optimal parameters consisted in using a frequency of 100 Hz and 100 s-long treatments. The implication of clathrin-mediated endocytosis was determined, and it was shown that high cell viability levels can be preserved after plasma treatment. In this study, optimization of the plasma delivery protocol was pursued to further understand substance internalization processes and to try to enhance its efficiency. Plasma-induced uptake and its kinetics in both HeLa and 4T1 cells was investigated. Direct and indirect plasma treatments as well as the implication of reactive species in plasma induced internalization were also compared. Finally, the delivery of an anti-cancer drug was assessed.

2. Materials and methods

Chemicals. All chemical reagents otherwise stated were purchased from Sigma-Aldrich. Cell culture reagents were obtained from GIBCO (ThermoFisher scientific).

Plasma Gun setup. Helium plasma was generated with a Plasma Gun (Robert et al., 2009). This system is a plasma jet based on a dielectric barrier discharge (DBD) reactor with a coaxial geometry powered by microsecond voltage pulses. High purity helium gas (Alphagaz 1, Air Liquide) was flushed at 0.5 slm through the reactor. Plasma is ignited by applying high voltage pulses across the two electrodes. Gas flow rate was controlled by a digital mass flow meter (red-y compact series, Vögtlin Instruments). As shown in Fig. 1, the DBD reactor consists of an inner high voltage hollow electrode (2 cm long, inner diameter 0.8 mm) and an outside grounded ring electrode set on the outer surface of a borosilicate capillary. A single voltage pulse, with both rise time and fall with at half maximum of 2 μ s, having a peak amplitude of 14 kV and delivered at 100 Hz or 1,000 Hz was used. The voltage polarity was set positive. Plasma generated in the reactor, then propagated in the capillary before reaching ambient air. The dielectric capillary was 12 cm long and tapered in the last 2 cm (lower part), the inner diameter was 4 mm in the upper part and 1.4 mm in the lower part, glass thickness was 1 mm. All experiments were conducted with a capillary-target distance of 1 cm.

Cell culture. 5×10^4 HeLa (Human cervical cancer cells, ATCC® CCL-2™) and 4T1 cells (murine breast cancer cells, ATCC® CRL-2539™) were seeded in the center of 24-well plates (Sarstedt polystyrene plate; 83.3922.005) to reach full confluence after 24 h. In order to achieve a seeded area corresponding to a circle of 6 mm in diameter (around 28 mm²), droplets of 100 μ L containing cells were carefully set in the center of the well. Plates were incubated overnight in this configuration. HeLa cells were grown in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (P/S), 1% GlutaMAX™ and 1% Non-essential amino acids. Roswell Park Memorial Institute (RPMI) medium supplemented

with 10% FBS and 1% P/S was used for 4T1 cells. Medium was changed every two to three days, cells were detached with trypsin twice a week and were incubated at 37 °C in a humidified atmosphere containing 5% CO₂. Cells were routinely tested for mycoplasma infection and all experiments were done with mycoplasma-free cells (MycAlert™, Lonza).

Cell membrane internalization analysis. Propidium iodide (PI, 668 Da) and FITC-Dextran (4 kDa, 70 kDa and 150 kDa) were used as molecular markers for cellular uptake and they were used at 0.05 mg/mL and 1 mg/mL, respectively. Both PI and FITC-Dextran were added to each well right before plasma treatment (stock PI solution at 0.5 mg/mL and stock FITC-Dextran solution at 10 mg/mL). Molecular uptake was assessed either just after treatment or after 30 min of incubation of the cells in their treated medium at 37 °C. The percentage of cells with an efficient uptake was measured by flow cytometry (Becton Dickinson, LSRII) and corresponds to the percentage of positive cells in all experiments; PI fluorescence was acquired in FL-2 channel. For each condition 10,000 events were collected in the gate corresponding to both cell lines. For fluorescence imaging and probe uptake visualization, cells were observed by microscopy using an inverted microscope Axio Observer.Z1 (Zeiss) equipped with a 10 \times objective (Plan-Neofluar 10x and 40x, Zeiss), pictures were acquired on Zen 2 Software, Zeiss) for both PI and FITC-Dextran. After plasma treatment and incubation in the treated medium following the usual protocol described above, cells were fixed with a solution of PBS containing 2% paraformaldehyde (PFA) during 30 min at 37 °C prior imaging. All images were taken with the same exposure time for the sake of comparison.

Direct plasma treatment. Cells were treated 24 h after seeding. They were washed with phosphate buffer saline (PBS) and were, in the baseline protocol, treated in Opti-MEM™ culture medium supplemented with 1% FBS and 1% P/S (200 μ L per well). The culture plate was positioned on a grounded metallic plate during plasma treatment. Capillary-to-cells distance was set at 11 mm (capillary-to-medium surface was around 10 mm). To assess the impact of the type of medium, HeLa cells were treated by plasma in Opti-MEM™ supplemented with either 1% FBS or 10% FBS or in MEM™ supplemented with 10% FBS. The influence of volume was determined by varying the volume of medium (200, 300 or 400 μ L) in which the cells were treated.

Indirect plasma treatment; plasma activated medium (PAM). PI was diluted in Opti-MEM™ medium at 0.05 mg/mL, this solution was treated by plasma (100 Hz, 100 s, 14 kV). Thereafter, cells were incubated with this treated medium for 30 min at 37 °C followed by flow cytometry analysis.

Substance uptake kinetics. Uptake kinetics were measured by adding PI after plasma treatment while keeping the 30-minute incubation at 37 °C. PI was added either right after treatment or upon 1, 3, 5, 10, 15, 20, 25 or 30 min. Internalization was assessed by flow cytometry in HeLa and 4T1 cells and by fluorescence microscopy for 4T1 cells. The fluorescence microscopy analysis of treated HeLa cells is available in a previous paper (Vijayarangan et al., 2017).

Cell viability assay. Cell viability was assessed using the XTT assay kit (Cell Proliferation Kit II, Roche) 24 h after direct and indirect plasma treatment. Absorbance was measured with spectrophotometry at 450 nm and 650 nm (Victor³ V). The cell viability was calculated as the ratio of the absorbance of treated cells over untreated control, this ratio was normalized as a percentage. All calculations and procedures were performed according to the manufacturer's protocol.

Doxorubicin drug delivery. Doxorubicin was diluted in Opti-MEM™ medium at 10 mM. 4T1 cells were incubated in Doxorubicin solution and treated by plasma with indicated parameters. After 30 min of incubation at 37 °C, the amount of intracellular delivered doxorubicin was assessed by flow cytometry (FL-1 channel). Doxorubicin-only condition corresponds to the control.

Stress fiber evidence. HeLa cells were seeded one day before the plasma treatment. The induction of stress fibers was investigated by labelling actin filaments with FITC-phalloidin. After plasma treatment, cells were rinsed using PBS and fixed with 2% p-formaldehyde (PFA)

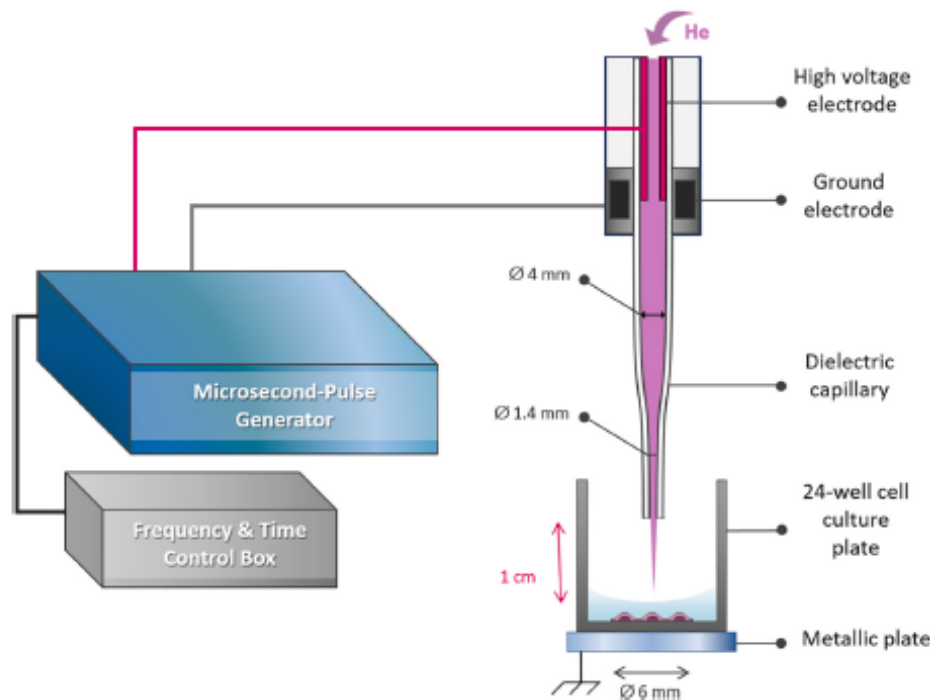


Fig. 1. Scheme of the Plasma Gun setup for direct plasma treatment.

20 min at room temperature. Cells were then rinsed and incubated with 0.1% Triton X-100 in 2% PFA for 15 min at room temperature and washed twice. Phalloidin (1:100 at 5 $\mu\text{g}/\text{mL}$, diluted in PBS) was added thereafter and cells were incubated at room temperature for 40 min. Cells were then rinsed three times and two drops of Vectashield[®] mounting medium was applied on the wells (after removing PBS) right before imaging. Fluorescence imaging was performed using an inverted microscope (described above).

Statistical analysis. Unless otherwise indicated, each experiment was performed in triplicates with two independent repeats. All numerical data are expressed as mean \pm standard deviation. Statistical analysis was performed using GraphPad Prism version 6.07 (GraphPad software) or Microsoft Excel. Differences in results were considered statistically significant when p -values were lower than 0.05 (*), 0.01 (**), or 0.001 (***). Any p -value greater than 0.05 was considered statistically not significant.

3. Results

3.1. Direct plasma treatments

3.1.1. Plasma conditions adjustments

In a previous study (Vijayarangan et al., 2017), it was found that 10,000 pulses (frequency at 100 Hz and treatment time of 100 s) was the best condition for combining efficient uptake and low toxicity in HeLa cells. Fig. 2. a. show the gating used for flow cytometry analysis. The percentage of PI positive cells was higher in plasma treated condition (13.65%) compared to the control condition (7.82%). In this study, we further explored the impact of medium composition and volume on the efficiency of cellular internalization. The impact of the treatment volume in molecular uptake was investigated (Fig. 2.b.). Interestingly, highest uptake was achieved using 200 μL of medium (17.04% \pm 2.4 of PI positive cells) compared to 300 and 400 μL (6.3% \pm 1.9 and 6.0% \pm 2.1 respectively). These two latter values are

not significantly different to that obtained for the control condition (5.9% \pm 0.9). According to those data, all following experiments were done using 200 μL of Opti-MEM[™]-1% FBS. Next, HeLa cells were treated in Opti-MEM[™] supplemented with either 1% or 10% FBS. As shown in Fig. 2.c., when cells were treated in Opti-MEM[™] containing 10% FBS, the percentage of PI-positive cells was significantly lower compared to cells treated in the same medium but containing only 1% FBS (9.1% \pm 2.0 versus 16.4% \pm 1.9). Similar values were obtained when the medium was switched to MEM supplemented with 10% FBS suggesting that this effect is likely due to the FBS content. These results indicate that the configuration using Opti-MEM[™]-1% FBS seems to be a better condition compared to the usual 10% FBS one. Plasma treatment made in medium containing 10% FBS as performed in some studies (Leduc et al., 2009) is less efficient.

3.1.2. Assessment of cellular uptake induced by plasma treatment on 4T1 breast cancer cell monolayer

In a previous study made with HeLa cells, we measured some cell detachment in the plasma-impacted area (Vijayarangan et al., 2017). Uptake efficiency was assessed in 4T1 cells, as they show more resistance against mechanical stress and toxicity (Pillar et al., 2018). This efficiency was assessed by fluorescence microscopy using several FITC-dextran sizes. Fig. 3. shows the cells in the plasma impact zone after treatment and a 30-minute incubation at 37 $^{\circ}\text{C}$. Plasma-induced cell monolayer disruption was much lower in 4T1 as shown by limited cell detachment as compared to that observed in HeLa cells measured previously (Vijayarangan et al., 2017). Internalization efficiency decreased with the size of the molecule as shown by the decrease in fluorescence observed. 4- and 70-kDa FITC-Dextran were successfully delivered into 4T1 cells after plasma treatment and 150 kDa FITC-Dextran as well but to a lower extent. Other studies such as Leduc et al. (Leduc et al., 2009) also reported the use of small fluorescent dextrans. Plasma induced uptake therefore seems effective for such small molecules and more challenging for higher molecular weight compounds

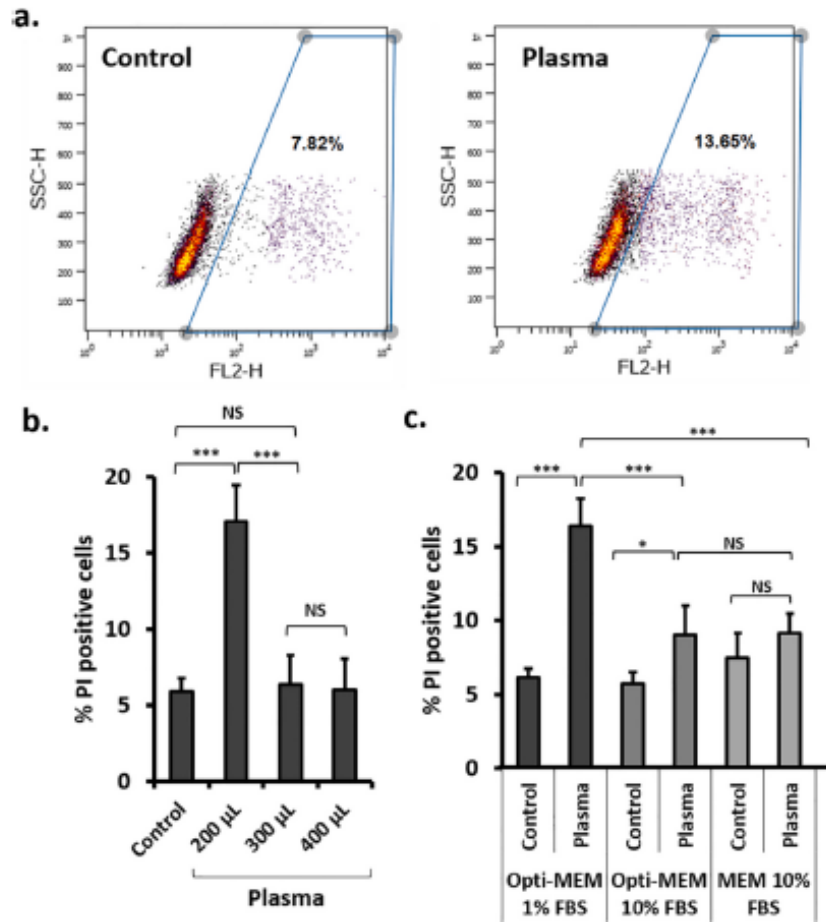


Fig. 2. a. Example of gating for PI positive cells for control and plasma conditions. b. Influence of serum content and medium volume on HeLa cellular uptake efficiency. Cells were treated in 200 µL, 300 µL or 400 µL of Opti-MEM-1% FBS. c. Influence of treatment volume on HeLa cellular uptake. Cells were treated in either Opti-MEM or MEM containing indicated percentage of FBS. Plasma parameters used are 10000 pulses (frequency at 100 Hz for 100 s at 14 kV).

and chemicals such as plasmids.

3.1.3. Uptake kinetics

All previous plasma treatments involved the addition of the molecule of interest (i.e. PI or FITC-Dextran) before plasma treatments. Previous studies involving electroporation reported that when the molecule was added after treatment, the efficiency decreased according to the time of the molecule addition (Gothelf et al., 2003). The influence of the time of addition of PI on uptake efficiency was investigated. By contrast to electroporation, data indicated that the internalization efficiency was increased when PI was added after plasma treatment. Fig. 4. a. shows that the uptake efficiency peaked when PI was added 3 min after the treatment ($39.3\% \pm 2.9$ of PI positive cells compared to $18.0\% \pm 3.4$ when PI is added before treatment). Then, the percentage of positive cells slowly decreased until it reached the control condition ($4.3\% \pm 2.3$) when PI was added after 30 min. As first hypothesis, we considered that PI might have been degraded in the plasma treated medium which could explain the higher fluorescence intensity when PI is added post-plasma treatment. To check whether this observation could be due to a degradation artifact, we measured the fluorescence intensity of PI solutions treated by plasma and no molecular damage was measured (Fig. S1. a.). These results show that plasma-induced internalization does not seem to follow the same process as

electroporation. The trend of the percentage of positive cells i.e. a peak after 3 min and then a continuous reduction illustrates the non-toxicity of the plasma treatment. Indeed, after a 100 s-plasma treatment coupled with a 30 min-incubation, cell integrity is restored as PI uptake is restricted. Viability measurements in a previous study also confirms these results (Vijayarangan et al., 2017). It is worth indicating that with these parameters and treatment conditions, cell viability was not too much impacted (~70 to 100% versus non-treated cells).

When cells were incubated at 4 °C (Fig. 4. b.), the uptake efficiency severely decreased; the highest value of PI positive cells is $7.2\% \pm 1.4$ compared to $33.1\% \pm 4.2$ when cells were incubated at 37 °C. These results are similar to that observed in electroporation which is also highly impaired by low temperature (Diaz-Rivera and Rubinsky, 2017). As clathrin-mediated endocytosis was found to be active upon plasma treatment (Vijayarangan et al., 2017), this pathway is severely inhibited at 4 °C.

In a second step, we evaluated if similar data could be obtained with 4 T1 cells. As shown in Fig. 4.c., the percentage of PI positive cells was also higher when it was added after treatment but uptake efficiency peaked sooner, i.e. right after treatment, while it was optimal 3 min after plasma treatment for HeLa cells. When PI was post-added 1 min, the percentage of PI positive cells was $26.7\% \pm 3.2$ close to $22.7\% \pm 3.0$ when PI was added before the treatment. It is also

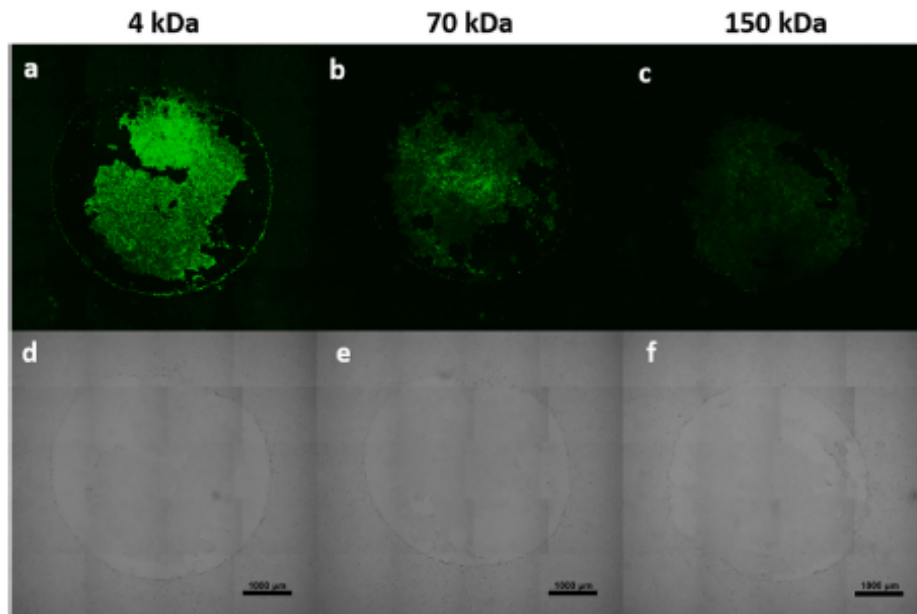


Fig. 3. Fluorescence microscopy images of 4T1 cells treated with plasma (100 Hz - 100 s - 14 kV) in presence of 4 kDa (a, d), 70 kDa (b, e) or 150 kDa (c, f) FITC-dextran and after a 30-min incubation time. Upper part: fluorescence images; Lower part: brightfield images.

important to note that contrary to HeLa cells, 4T1 cells have less if any cell detachment (Fig. 4. d.). In Fig. 4. d., fluorescence microscope images indicate that when PI was added after plasma treatment, a circular impact zone (visible uptake area) was observed in all conditions.

3.1.4. Induction of stress fibers

We used phalloidin to assess whether there were evidences of stress fibers into HeLa cells after plasma treatment. Stress fibers are contractile bundles composed of actin, myosin and different crosslinking proteins. These fibers have been shown to play an important role in cell morphogenesis, adhesion, and migration (Burrige and Wittchen, 2013). After plasma treatment following the usual protocol, stress fibers were observed in cells while no stress bundles were found in the control condition nor helium (gas flow only) or PAM conditions (Fig. 5.). This could be produced by the mechanical stress induced by plasma. Stress fibers were mainly observed for cells located at the boundary of the detachment zone, i.e. on the edges of the plasma impact zone. Stress fibers were almost not detected for cells located at larger radial distance from this impact zone, i.e. more than about one mm away from the plasma jet axis. The measurement of stress fibers for HeLa cell probably correlates with how plasma affects the cells and their detachment.

3.2. Influence of the RONS and electric field on plasma-induced internalization

3.2.1. RONS implication

We investigated whether the RONS produced by plasma were involved in cellular uptake in both HeLa and 4T1 cells. We proceeded by treating the cells indirectly with plasma-activated media (PAM). In these conditions, cells were only exposed to the long-lifetime reactive species produced by plasma without the impact of the electric field. As observed in Fig. 6. a., efficient uptake was achieved when HeLa cells were incubated for 30 min at 37 °C with PAM.

Even if about same percentages of PI positive cells were measured for direct and indirect plasma treatments ($19.4\% \pm 2.5$ for direct plasma and between $13.7\% \pm 3.6$ and $21\% \pm 4.0$ for indirect

treatments), the much lower detachment level associated with PAM treatment results in a higher uptake efficiency of cells with direct treatment as only non-detached cells are considered. RONS produced by the plasma, either in direct or indirect protocol, play a key role in HeLa cell membrane internalization, there was no statistically significant difference between each condition. Similar experiment was conducted with 4T1 cells as documented in Fig. 6. b. Interestingly, PAM was not sufficient to induce cellular uptake; the percentage of PI-positive cells was not statistically different to control cells ($7.2\% \pm 2.6$ for the control compared to values between $5.9\% \pm 3.2$ and $8.3\% \pm 3.8$ for the PAM conditions).

The effect of PAM on HeLa cell viability was assessed (Fig. 7.). There was no impact on the cell viability one day after PAM treatment, all values were equal or above 100%. It means that for this cell line, while very minor cell damage was measured, efficient uptake was achieved with RONS only exposure. Note that the cell viability with direct plasma treatment was ranging from 70 to 100% (Vijayarangan et al., 2017).

3.2.2. Electric field implication in plasma-mediated internalization

The role of the electric field on cellular uptake was assessed. For this purpose, HeLa cells were treated with plasma electric field only, using a configuration described in a previous paper (Vijayarangan et al., 2017). In this arrangement, cells seeded in culture plates were exposed to plasma electric field by means of an inverted setup in order to treat the bottom of the wells with plasma. A previous study showed that plasma electric field alone was not sufficient to induce cellular internalization. An additional experiment was performed, involving the combination of first electric field alone exposure followed by PAM addition. PAM was prepared and added at the optimal condition for cellular uptake, i.e. with a 3 min delay as inferred from data in Fig. 4.a. No detachment but no significant uptake as well were measured (data not shown). All those results indicate that direct plasma treatments were still more efficient than RONS alone, electric field alone or RONS-electric field combination treatments.

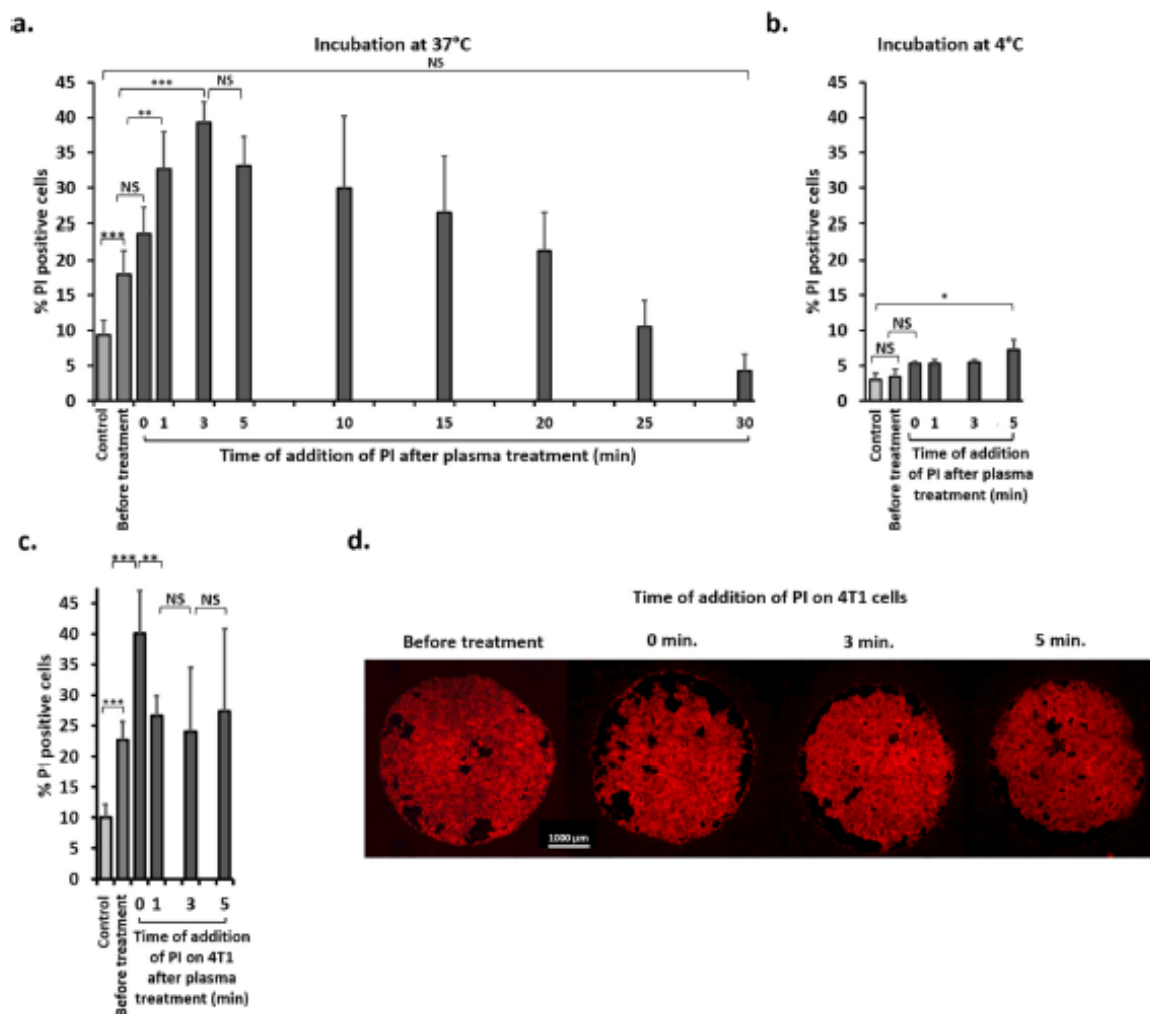


Fig. 4. a. and b. PI uptake kinetics for HeLa cells. c. and d. PI uptake kinetics for 4T1 cells. d. Visualization of the plasma impact zone, PI was added in the cell medium either before treatment, straight away (0); 3 or 5 min after treatment. Cells were treated with 100 Hz - 100 s - 14 kV plasma conditions. PI was added before or after treatment at different times (from 0 to 30 min). Cell incubation at 37°C (a, c. and d.) and 4°C (b).

3.3. Drug delivery

Molecular internalization experiments with direct plasma treatments were conducted using Doxorubicin (DxR), a well-known anticancer drug, in 4T1 cells (Fig. 8). Uptake was more efficient when DxR was added after plasma treatment. When DxR was post-added, percentages of PI positive cells were above 25%, whatever the time delay of cell exposure after PAM preparation, compared with the addition of the molecule prior treatment ($13.0\% \pm 3.7$). DxR integrity was also assessed by treating DxR solutions by plasma and no decrease in molecule fluorescence was measured (Fig. S1. b).

4. Discussion

In this work, we first investigated both the role of the volume and of the nature of the cell medium on the plasma-induced uptake (results presented in Fig. 2. b. and 2. c.) whilst on the basis of a previous work (Vijayarangan et al., 2017), other parameters were kept constant, i.e. voltage, pulse repetition rate, distance to the wells and gas flow. As expected, in line with previous studies of RONS production [e.g.

Graves, 2014; Bauer, 2019; Khlyustova et al., 2019]17, 28–29], the use of a larger volume results in the decrease of the RONS concentration thus lowering the uptake efficiency (Fig. 2. b.). As can be seen in Fig. 2. c., the role of the cell medium is of crucial importance leading to huge variation in the percentage of PI positive cells, indicating that the efficiency is highly dependent on the type of medium, especially the presence of FBS. It is quite well known that FBS, by its composition, can act as an antioxidant (Zhang et al., 2016). Increasing the FBS content in either Opti-MEM or MEM medium resulted in a drastic decrease cellular uptake efficiency upon plasma treatment, revealing the potent antioxidant action of this compound (Fig. 2. c.). Antioxidant content conventionally used during *in vitro* experiments can fully prevent the cell uptake process, which may then hinder one of the mode of action of plasma treatment when translating to *in vivo* or clinical protocols.

Plasma-induced uptake previously documented for HeLa cells (Vijayarangan et al., 2017) was confirmed in the present work for the 4T1 cells, using a limited number of plasma pulses delivered during 100 s at 100 Hz repetition rate. Under this condition, 4T1 cells molecular uptake leads to much less detachment in comparison with that observed for HeLa cells, and it is achieved with a very high cell

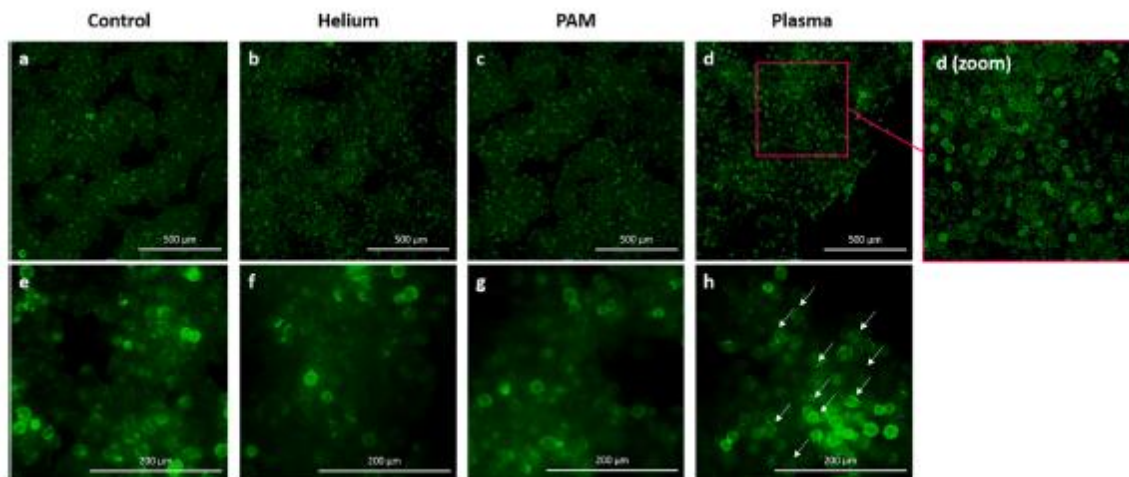


Fig. 5. Actin fibers cell imaging in HeLa cells in control condition (a., e.), helium (b., f.), PAM (c., g.) and plasma condition (d., d.(zoom), h.). Imaging with 10x and 40x objectives. Plasma treated condition (100 Hz – 100 s – 14 kV).

viability. The ability of plasma treatment to successfully allow substance uptake by 4T1 cells without impacting the cell viability is worth noticing as those cells are amongst cell lines that are known to be quite resistant for both chemotherapy treatment and transfection. 4 kDa and 70 kDa FITC-Dextran were successfully delivered into 4T1 cells after plasma treatment and the 150 kDa one to a much lower extent (Fig. 3). If correlated to hydrodynamic radii, these molecules have 1, 6 and 8 nm radius for 4, 70 and 150 kDa FITC-dextran, respectively (Armstrong et al., 2004). Therefore, molecules having a radius larger than eight nm seem to be hardly taken up by 4T1 cells following plasma treatment in our experimental conditions. Note that similar observation was made for HeLa cells (Vijayarangan et al., 2017). Other studies such as Leduc et al. (Leduc et al., 2009) also reported on the use of small fluorescent dextrans. Plasma-induced internalization therefore seems effective for such small molecules and more challenging for higher molecular weight compounds and chemicals such as plasmids. While not reported in this work, recent studies have shown that PAM combined with pulsed

electric field can also not only allow for a higher internalization rate (percentage of positive cells) but also to an increase of the cell uptake (density of permeant molecule per cell) (Chung et al., 2020).

The main outputs from this work were inferred from the uptake kinetics study for both cell lines. For the first time to the best of our knowledge, we found that an enhanced uptake was obtained when the fluorescent probe was added with some delay after plasma exposure. For 4T1 cells, the highest uptake efficiency was measured when PI was added right after plasma delivery. The internalization rate is kept almost constant for PI injection delays ranging from one to five minutes (Fig. 4. c.). For the setting corresponding to the addition of the probe right after plasma exposure, the internalization rate is roughly doubled in comparison with that achieved when PI was added before plasma exposure. This impact of the delay on the uptake rate was also observed for doxorubicin, a well-known antitumor drug (Fig. 8.). The uptake of 4T1 cells was doubled when the drug was added from 1 to 5 min post-exposure. This data is quite similar to that obtained with PI and

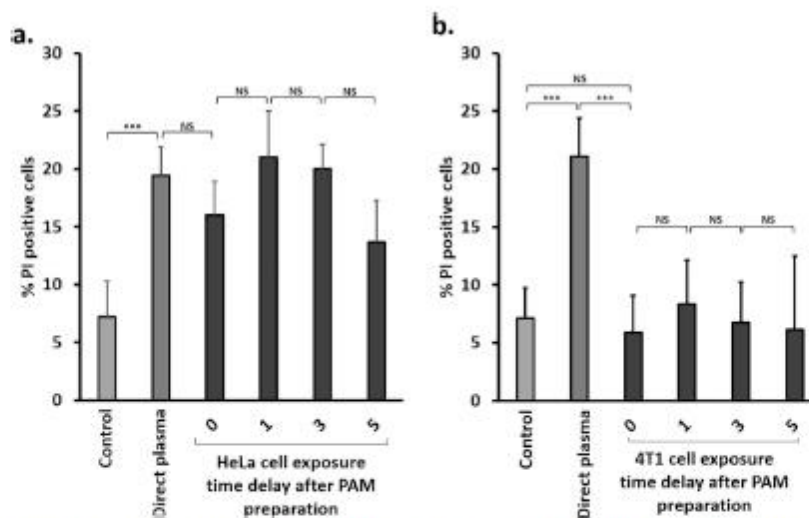


Fig. 6. Cellular uptake kinetics of HeLa cells (a.) and 4T1 cells (b.) with indirect plasma treatment for different cell exposure time delays (0, 1, 3 and 5 min) after PAM preparation. Uptake measured with direct plasma treatment (PI injected before treatment) is plotted for the discussion. Plasma condition: 100 Hz - 100 s - 14 kV.

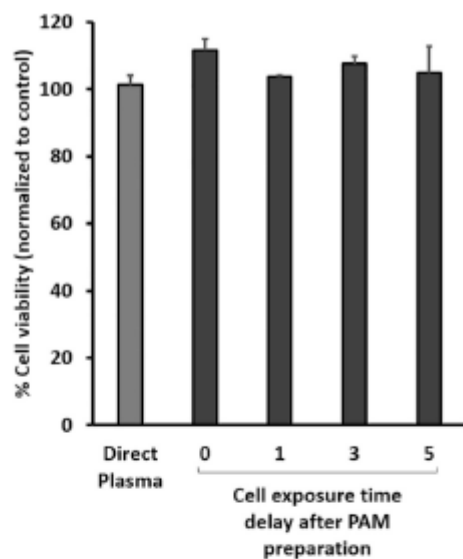


Fig. 7. Viability of HeLa cells 24 hours after PAM exposure. PAM was added at 0, 1, 3 and 5 minutes after its preparation. Plasma condition: 100 Hz - 100 s - 14 kV.

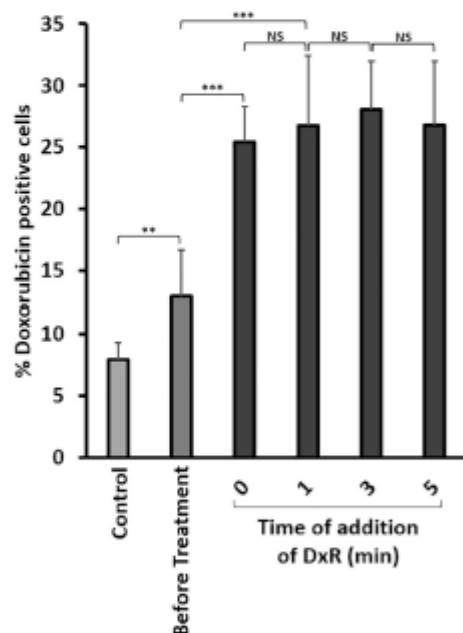


Fig. 8. Drug delivery in 4T1 cells using plasma. 10 mM Doxorubicin (DxR), control condition and kinetics. Plasma condition: 100 Hz - 100 s - 14 kV.

coherent considering the fact that DxR and PI are two small molecules having the same molecular weight range (543.4 g/mol and 668.39 g/mol, respectively) and similar planar structure. Data obtained with HeLa cells show as well that adding PI post-exposure is beneficial for the molecule uptake (Fig. 4. a.). The increase is not as sharp as that obtained with 4T1 cells, there is a gradual increase of uptake rate with the doubling of this rate obtained when PI was added 3 min post-exposure. Then, there is a gradual decay of the rate of positive cells when

PI was added after 3 min and the percentage of positive cells became similar to that of control cells after 25 min. Those data suggest that in HeLa cells, the uptake induced by the plasma exposure is quite long lasting. It is worth notifying that the lower uptake efficiency measured when the PI was added before plasma exposure was not due to the fluorescent probe alteration by plasma, during the plasma treatment, and the plasma generated RONS, during the cell incubation. We checked that PI was not degraded by measuring its fluorescence intensity upon different plasma treatment (Fig. S1 a.).

The results presented in this paper indicate first that plasma treatments induce a transient plasma membrane destabilization leading to an efficient drug uptake. Second, this destabilization lasts up to tens of minutes allowing an efficient uptake of extracellular substance of small size such as PI and DxR at different time's post-treatment. Moreover, this destabilization did not induce a severe cytotoxicity as confirmed by the viability assay made on both cell lines after direct plasma treatments. Such difference has been reported in pulsed electric field reversible electroporation experiments both *in vitro*, *in vivo* and in clinics. For these electroporation studies, the electric field exposure can be as short as few or few tens of nanoseconds; most of the time it consists in a few pulses delivery at low frequency, typically one or few Hz, while drug uptake was shown to be active for minutes long duration after the treatment. This feature has been accounted for translation to clinical therapeutic protocol setting (Mir et al., 2006) where electrostimulation also results in a transient blood flow modulation in the region where pulsed electric fields are applied (Bellard et al., 2012). Coming back to plasma-induced uptake and in the perspective of *in vivo* or clinical experiments, our results indicate that drug injection must be planned by taking into consideration the long time persisting membrane permeability triggered by the short time plasma exposure. In addition, it was also reported that short duration plasma exposure may induced drastic blood flow increase and enhanced tissues oxygenation for tens of minutes after plasma treatment both in *in vivo* experiments (Collet et al., 2014) and in clinical study (Kisch et al., 2016).

When the experiments were conducted at 4 °C, the uptake efficiency severely decreased (Fig. 4. b.). This can be due to either a new balance among the different RONS concentrations, likely associated with different temperature-dependent reaction rates, or the cell membrane fluidity modulation at low temperature. No conclusion could be drawn on the implication of one or both of those processes. Nevertheless, as previously reported, the clathrin-mediated endocytosis was active upon plasma treatment (Vijayarangan et al., 2017) and this pathway is known to be severely inhibited at 4 °C. The impact of plasma exposure on the plasma membrane was reinforced with the formation of stress fibers (Fig. 5.). This is not surprising since as any physical trigger, plasma exposure could potentially induce a mechanical stress on the plasma membrane. *In vitro* studies from Hayakawa et al. (Hayakawa et al., 2012, 2011) showed that the actin filaments could also function as a mechano-sensor. The mechanical stress induced by plasma could trigger the activation of intracellular signaling molecules, such as MAP kinases, Akt, and PI3 kinase. Hence, the activation of specific signaling pathways potentially involved in cellular endocytosis and uptake.

Four different cell exposure protocols have been proposed and assessed to provide new insight on plasma uptake mechanistic: direct, indirect (so called PAM), electric field (EF) alone and electric field and consecutive PAM. Altogether, data obtained led to suggest and discuss one hypothesis to explain the role of drug injection delay with respect to plasma delivery. We hypothesized that that "plasma cell sensitization" could occur and plays a critical role for cell uptake. This sensitization may mainly involve EF and/or short living RONS. The role of long living RONS on this sensitization, during plasma exposure of 100 s, should be of minor influence as in any protocol 30-minute incubation was applied. Data from EF and consecutive PAM exposure experiments (exposed in section 3.2.), indicate that the combination of EF and long living RONS -as delivered with PAM, for which short-lived species are quenched within few second- to few tens of seconds- are not efficient for

such sensitization. The exposure of EF alone was also reported to be quite ineffective (Vijayarangan et al., 2017). Thus, we conclude that if relevant, the plasma cell sensitization, should imply both EF and short living RONS. PAM exposure alone was shown to be as efficient as direct plasma exposure for HeLa cells when PI was added before plasma treatment (Fig. 6.). These results show that, for HeLa cells, EF sensitization was not critical for the drug uptake. But, if PI was added after an optimized time, i.e. 3 min in our experimental conditions for HeLa cells, plasma sensitization would allow for a doubling of the uptake rate (Fig. 4. a.). Such a potential dramatic role of plasma sensitization would be even more drastic for 4 T1 cells for which PAM exposure was shown to be ineffective (Fig. 6. b.). It is worth mentioning that cell sensitization is also involved in pulsed electric field reversible electroporation where transient EF is described to account for so-called "pore" formation before reactive species and drug may interact with cell membrane (Rems et al., 2019; Van der Paal et al., 2019). Benefit from the combined treatment with direct plasma and chemotherapy reported for both *in vitro* (Körtzner et al., 2013) and *in vivo* (Brullé et al., 2012) experiments was also speculated to be associated with such plasma sensitization process. A previous study (Vijayarangan et al., 2017) showed that electric field (EF) treatments were not effective for an efficient substance uptake. The setup and configuration used to study the effect of the plasma EF was only used as a comparison tool to study the effect of the plasma components (as it might certainly not be the exact EF subjected to the cells in a direct plasma treatment). The hypothesis on why PAM alone was able to induce this uptake and not EF followed by PAM is that EF alone might sensitize the cells in a way that it prevents or inhibits the uptake induced by the long-term reactive species present in PAM solutions. EF treatments do not induce any toxicity 24 h after treatments as measured by XTT assay. A study from Lin et al. (Lin et al., 2017), provides interesting insights about how EF treatments can stabilize lipid rafts present on plasma membrane and how those rafts "are the primary sensing mechanism to external EF and regulate downstream protein signaling". Maybe, the raft reorganization into the lipid bilayer of the cells could prevent the action of PAM and reduce molecular uptake, which might explain why the PAM applied on the cells after EF treatment was not effective.

While suggested to be beneficial for cell uptake, plasma cell sensitization, i.e. plasma direct exposure with consecutive drug injection, also has some drawbacks. It was measured that direct plasma exposure induces low, but significant decrease of 24-hour viability in comparison with PAM exposure (Fig. 7.). Same holds true for EF alone or EF and consecutive PAM exposure impacts on cell viability (data not shown). Direct plasma exposure may also lead to severe cell detachment and then uptake rate decay for some cell lines, e.g. HeLa cells.

5. Conclusion

Uptake and uptake kinetics have been studied in detail for HeLa and 4 T1 cells. For the first time to the best of our knowledge, we demonstrate that an enhanced uptake rate was achieved when the chemical or drug was added with some delay after plasma exposure. For both cell lines, this led to a doubling of the uptake rate in comparison with previous protocol where the drug (PI or DxR) was added before plasma delivery. Our results indicate that plasma treatment induces a transient, quite long time persisting, and reversible action that leads to cell membrane uptake. The use of four protocols for plasma delivery, i.e. -direct, indirect (so called PAM), electric field alone and electric field and consecutive PAM- treatments, reveals a plasma-induced cell sensitization, implying both electric field and short living RONS. Such a plasma cell sensitization plays a key role for either cell line which can be positive with PAM exposure (e.g. HeLa cell in this work) or cells for which PAM treatment is almost inefficient (e.g. 4 T1 cells in this work). This work demonstrates the great potential of cold plasma for cell uptake and opens many new opportunities for combined protocols with electroporation and /or chemotherapeutic therapies.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijpharm.2020.119874>.

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