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Characterization of plasma mediated molecular delivery to cells *in vitro*

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ABSTRACT

Ion-based strategies have recently emerged as a method to facilitate molecular delivery. These methods are attractive as they separate the applicator from the treatment site avoiding some issues encountered with other electrically driven methods. Current literature on plasma delivery has shown utility *in vitro* and *in vivo* for both drugs and genes. To advance this technology more information must become available on the mechanism responsible for delivery and the effects of ion exposure on eukaryotic cells. This *in vitro* investigation found that molecular delivery facilitated by a DC-based plasma follows a dose–response behavior, with optimum uptake of Sytox Green occurring in two cell lines after 600 s of exposure. In both cell lines exposure to the discharge caused no adverse effects in viability for exposure times up to 600 s. It was also found that membranes treated with ions remained permeabilized for several minutes following plasma treatment and that membrane resealing exhibited first order kinetics.

1. Introduction

The application of electric fields using metal electrodes as contacts to tissue or media has been used extensively in molecular delivery to increase the localized effects of therapeutic molecules. This process is commonly referred to as electroporation or electropermeabilization. It has been suggested that electroporation works by creating a voltage bias across the cell membrane strong enough to induce dielectric breakdown (Weaver and Chizmadzhev, 1996). As a result the integrity of the cell membrane is compromised allowing therapeutic molecules to diffuse into the cell. Membrane destabilization has been shown to last up to several minutes after treatment in studies monitoring the influx of normally cell-impermeant tracer molecules. Transport of these molecules into the cell has been shown to follow first order kinetics with a rate constant of approximately 0.34 min^{-1} (Rols and Teissié, 1990; Neumann et al., 1998). The success of electroporation has been demonstrated by its extensive use in animal models to deliver molecules to cells in connective, epithelial, muscle and nervous tissue (Jaroszeski et al., 1999; Grossin et al., 2003; Weissinger et al., 2003; Kunieda and Kubo, 2004; Hirao et al., 2008; Yuan, 2008).

While electroporation is an effective technique for molecular delivery several drawbacks do exist. When used *in vivo*, electroporation has been shown to cause muscle stimulation and pain (Prausnitz, 1996; Denet et al., 2004). Also, exposure of cells or tissues to strong electric fields, especially at electrode–tissue interfaces, can lead to irreversible electroporation and ultimately cell death (Weaver and Chizmadzhev, 1996). Application of the electric field in a manner that avoids contact between the tissue and electrodes would enable delivery without the collateral damage seen with electroporation. One possible way of achieving this is by depositing ions onto a treatment site that have been generated with an atmospheric pressure direct current plasma.

Plasmas have been employed in biology to treat prokaryotic and eukaryotic cells. Application of plasma discharges to surfaces contaminated with bacteria has shown promise as a method for decontamination (Laroussi, 2000; Vleugels et al., 2005). It is thought that this technique works in a similar fashion to electroporation, where the microorganism's membrane is compromised (Moreau et al., 2008). Defects created in the membrane are thought to result from exposure to oxidizing components in the plasma and not from dielectric breakdown as is suggested with electroporation. Application of plasma to eukaryotic cells is a more recent field and has shown utility for augmenting molecular delivery. For example, corona discharges have been used to deliver tracer molecules and chemotherapeutic agents to cells *in vitro* (Ramachandran et al., 2008). High frequency discharges have been used to deliver plasmid DNA encoding GFP to neuronal cells *in vitro* (Ogawa et al., 2005). Finally, a nonequilibrium direct current plasma source has been successfully used *in vivo* to deliver plasmid DNA encoding luciferase to dermal cells in murine skin. In this experiment an intradermal injection of plasmid DNA was followed by the application of plasma directly to the outer surface of the skin. This study

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leads to measurable foreign gene expression for 30 days (Connolly et al., 2009).

The documented success of various ion sources to enhance molecular delivery suggests a validity of this novel technique. However, little is known regarding the mechanism leading to delivery and how these discharges affect the cell. The work presented here summarizes a series of *in vitro* experiments performed to determine the effects of plasma exposure on eukaryotic cells. This work examines the delivery efficacy, resealing kinetics and impact of plasma exposure on viability.

2. Materials and methods

2.1. Plasma generator

The plasma generator fabricated for this study was made from a 15 cm long Teflon dowel. This dowel was hollowed to create a tube having a 1 cm inner diameter and wall thickness of 2 mm. Two electrodes were positioned at either end of the 15 cm long tube. A steel hose barb attached to ground potential was positioned on one end of the tube to serve as a ground reference electrode and interface with a gas inlet stream. A flat annular stainless steel electrode with a 3.5 mm internal diameter occupied the opposite end of the Teflon tube. This electrode was connected to a high voltage direct current power supply (Spellman Model CZE200, New York, NY). A plasma discharge was created by applying an 8 kV potential to the annular electrode while a stream of ultra high purity helium (AirGas, Tampa, FL) was passed through the tube at a rate of 15 l/min. A software interface platform (National Instruments LabVIEW, Austin, TX) was used to control the high voltage power supply and allowed the voltage, maximum current and exposure time to be set prior to treatment. A current limit was set to 100 μA as a safeguard measure during treatment. Under operational conditions the generator provided a reading of 10 μA at 8 kV to the annular electrode and produced a visible glow discharge that measured 3.0 cm in length. A similar current was measured with an electrometer (Keithely Model 6517A, Cleveland, OH) on a 4 cm^2 stainless steel plate positioned 3.0 cm below the plasma generator.

2.2. Optical spectroscopy

Ultraviolet and visual (UV–vis) light spectroscopy was performed to determine the excited constituents in the discharge plume. Spectra were collected by placing a fiber optic probe (Ocean Optics QP230-1-XSR, Dunedin, FL) 0.5 cm perpendicularly from the plasma discharge. This probe was connected to a spectrometer (Ocean Optics USB2000) with a dispersion range of 200–1100 nm. Spectral information was recorded with a 5-s integration time and uploaded to a computer as a data matrix of wavelengths and intensities utilizing the manufacturer's software package (Ocean Optics SpectraSuite). This matrix was then analyzed in Matlab (The MathWorks, Natick, MA) with a peak detection algorithm. Peaks found with the program were then compared to those listed on the National Institute of Standards and Technology (NIST) Atomic Spectra Database.

2.3. Cell culture

A portion of this work was performed using B16F10 (ATCC CRL-6475) melanoma cells derived from C57Bl/6J mice. These cells were propagated in McCoy's 5A media (MediaTech, Herndon, VA) supplemented with 10% (v/v) fetal bovine serum (MediaTech) and 50 μg/ml gentamicin sulfate (MediaTech). Cultures were incubated at 37 °C in a 5% CO_2 atmosphere. Once cells were confluent on the culture surface they were subcultured at a 1:12 ratio.

The remainder of this work was performed with immortalized human keratinocyte cells, HaCaT (Cell Line Services, Eppelheim, Germany). These cells were propagated in Dulbecco's modified Eagle's media (MediaTech) supplemented with 10% (v/v) fetal bovine serum and 50 μg/ml gentamicin sulfate. These cells were also incubated at 37 °C in a 5% CO_2 atmosphere. Cells were subcultured at 90% confluency at a ratio of 1:6.

2.4. Tracer delivery

Treatments performed to determine the delivery capacity of the plasma discharge were carried out with B16F10 and HaCaT cell lines. Dual chambered tissue culture dishes (BD Falcon, Franklin Lakes, NJ) having an inner diameter of 15 mm and outer diameter of 60 mm were used to perform these experiments. Prior to treatment the inner chamber was loaded with 1×10^6 cells suspended in 500 μl of phosphate buffered saline (PBS). The outer chamber was filled with 2 ml of PBS and contained a 22-gauge tinned copper wire connected to ground through a 1.5 GΩ resistor. This ring prevented the plasma plume from being affected by air currents, which can change the nature of the discharge during treatments. The height of the generator was set at 3 cm, which was determined to allow the tip of the visible discharge plume to touch the cell solution in the inner well. Exposure times of 0, 120, 300 and 600 s were performed. After plasma exposure was complete 500 μl of 2 μM Sytox Green (Molecular Probes, Eugene, OR) was added to the cell suspension in each dish. Sytox Green is a fluorescent tracer molecule that is only able to enter cells having a compromised membrane. Once inside the cell this tracer molecule binds with nucleic acids which increases its fluorescence by >500 fold. After adding the nucleic acid probe, cells were incubated at 37 °C and 5% CO_2 for 45 min. Following incubation, cells were resuspended by pipetting and three representative samples of 150 μl were transferred to a black 96-well spectroscopy plate (Corning, Lowell, MA). Fluorescence levels were quantified with a fluorescent plate reader (BioTek Instruments FLx800, Winooski, VT) using a 485 nm excitation filter and 528 nm emission filter. Each treatment condition was performed a total of 3 times per experiment and each experiment was repeated 3 times in order to increase the statistical power.

2.5. Viability assay

Treatments performed to determine cell viability following plasma treatment were also carried out with both B16F10 and HaCaT cell lines. Dual chambered tissue culture dishes, as described, were also used for this study. Before treatment the inner chamber of the dual dish was loaded with a suspension of 1×10^6 cells suspended in 500 μl of supplemented growth media. The outer chamber of the dish contained the 22-gauge wire immersed in 2 ml of PBS which was connected to ground through a 1.5 GΩ resistor. An operating generator height of 3 cm was used with exposure times of 120, 300 and 600 s. Following exposure, the cell suspensions were incubated at 37 °C and 5% CO_2 for 4 h. After incubation 50 μl of trypan blue (MP Biomedicals, Solon, OH) was added to the cell suspension. This suspension was then pipetted into a dual chambered hemocytometer (Hausser Scientific, Horsham, PA), and a dye exclusion viability assay was performed by light microscopy. This procedure was repeated a total of 3 times per treated sample. Each treatment condition was performed a total of 3 times per experiment and each experiment was repeated 3 times.

2.6. Membrane resealing

Kinetic experiments were performed to determine the time after plasma treatment when tracer molecules were no longer able to enter the cell. These experiments were carried out by adding

1×10^6 B16F10 or HaCaT cells suspended in 500 μl of unsupplemented media to a dual chambered tissue culture dish. As described above, the outer chamber of the dish contained a 22-gauge wire, immersed in 2 ml of PBS, that ran through a 1.5 GΩ resistance to ground. The generator was set 3.0 cm above the fluid level and cells were exposed to plasma for a total of 600 s. After exposure 500 μl of 2 μM Sytox Green was added to the cell suspension. The suspension with the tracer molecule was mixed by pipetting and three representative samples of 150 μl were transferred into a 96-well spectroscopy plate. Fluorescence levels were obtained at 60 s intervals for a total of 20 min with a fluorescent plate reader using a 485 nm excitation and 528 nm emission filter set. Due to processing time the first readings occurred 120 s after the dye was added to each well. Each treatment was performed a total of 3 times per experiment and each experiment was repeated 3 times in order to increase the statistical power.

2.7. Statistical analysis

After gathering data from delivery, viability, and resealing experiments the mean and standard error were calculated for each identically treated group. Using the obtained information a series of Student's *t*-tests were performed with a confidence level of 95%. This allowed relative comparisons to be made concerning the implications of each treatment time.

3. Results and discussion

The plasma generator fabricated for this study created a stable discharge and was capable of operating in a reproducible manner. This generator operated with a potential of 8 kV on the high voltage annular electrode and a helium flow rate of 15 l/min. Operating under these conditions the generator supplied an average current of 10 μA to the high voltage electrode, which corresponded to a maximum power consumption of 80 mW by the plasma discharge. The visible plume created by this discharge measured approximately 3 cm in length. Infrared temperature measurements (Fluke model 62, Everett, WA) made at 0.5 cm intervals throughout the visible discharge showed no thermal variations from that of the flowing gas alone. This suggests that the visible plume is an afterglow and not an active arc or plasma volume. Fig. 1 shows the plasma generator operating 3 cm above a tissue culture dish.

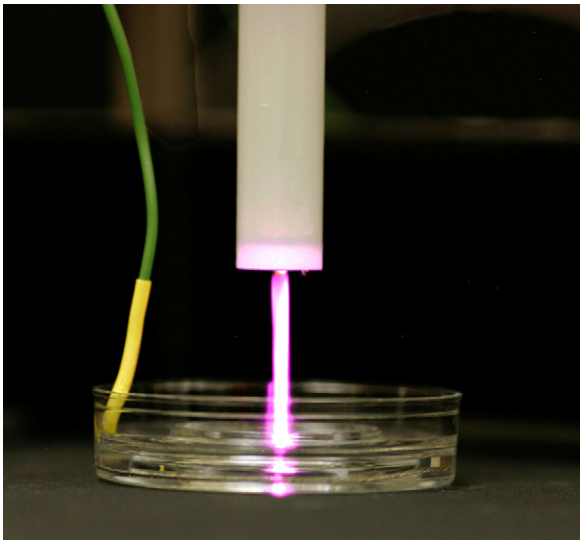

Fig. 1. Plasma generator positioned 3 cm above a tissue culture dish with an ionized stream of gas being directed into the inner well. The outer chamber of the tissue culture dish contains a 22-gauge wire connected to ground through a 1.5 GΩ resistor.

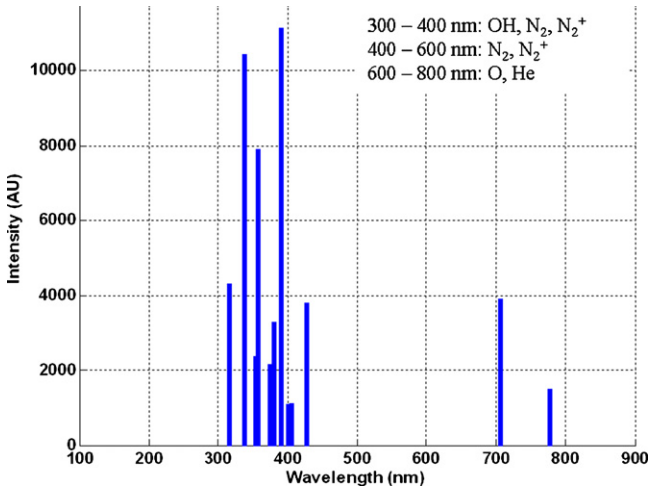

Fig. 2. Peak detection results from a spectrum collected at the opening of the generator with a 5-s integration time.

Ultraviolet and visual light spectroscopy was used to determine possible excited constituents produced by the plasma generator. Fig. 2 shows a spectrum collected from the discharge at the opening of the generator. Emission lines in the range of 300–400 nm correspond to OH, N_2 and N_2^+; 400–600 nm to N_2 and N_2^+; and 600–800 nm to O and He (Laroussi and Lu, 2005). The helium excitation line prevalent in the spectroscopic data occurs at 706.5 nm corresponding to the 1s2p–1s3s atomic configuration (Kang et al., 2002). Peaks from species other than helium are suggested to result from energy transfer to molecules from ambient atmosphere at the opening, plasma region, of the generator.

Molecular delivery experiments were performed with HaCaT and B16F10 cells to determine the ability of plasma to enhance delivery of small molecules *in vitro*. These experiments were performed using the setup discussed in the Section 2.4. Cell suspensions were treated with the plasma discharge for 120, 300 and 600 s. After exposure a fluorescent nucleic acid stain was mixed with the cell suspension to quantify the extent of delivery attainable at each treatment time. Fig. 3 shows cells stained with the nucleic acid probe Sytox Green. Fluorescent values of HaCaT cells following delivery had a mean rise of 13% for 120 s, 41% for 300 s and 62% for 600 s relative to untreated control samples. Similar increases of 7%, 21% and 55% were obtained when B16F10 cells were plasma treated for 120, 300 and 600 s, respectively. Fig. 4 displays these fluorescence quotient data for B16F10 and HaCaT cells, which were obtained by dividing the fluorescence value of the treated sample (F) by the respective control value fluorescence (F_0). Statistical analysis performed on these data concludes at the 95% confidence level that HaCaT and B16F10 cells exposed to 300 and 600 s had a mean fluorescence higher than that of their respective control groups.

The next set of experiments ascertained the effect plasma treatment had on cell viability. In these experiments HaCaT and B16F10 suspensions were again exposed to the plasma plume for 120, 300 and 600 s. Immediately following treatment the cell suspensions were incubated at 37 °C and 5% CO_2 for 4 h. Trypan blue assays were then performed to determine cell survival following plasma exposure. Initial HaCaT cell viability was 96.5%, which decreased in the control sample to 92.7% after processing the cells for treatment and incubation. Cells from the treatment groups had a viability of 90.5% for 120 s plasma exposure, 93.0% for 300 s and 90.8% for 600 s. B16F10 cells had an initial viability of 96.0%, which decreased to

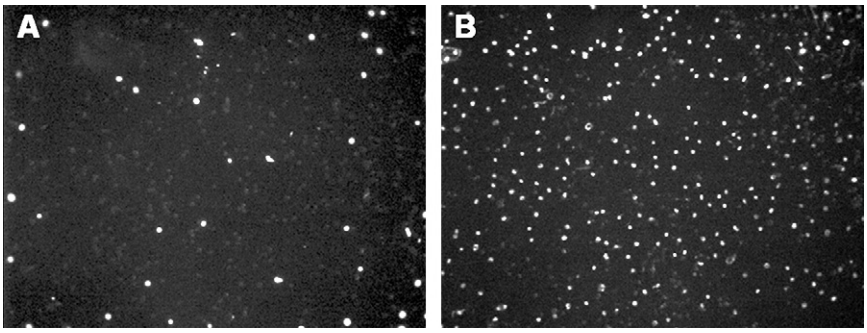

Fig. 3. Fluorescent images of cells incubated with Sytox Green for 45 min at 37 °C and 5% CO_2. (A) No exposure to plasma prior to incubation. (B) Six hundred seconds of plasma exposure prior to incubation.

90.2% in the control group 4 h later. Treatment group viability was found to be 88.9% for 120 s, 87.9% for 300 s and 89.2% for 600 s. Results are summarized in Fig. 5. Analysis of these data conclude at the 95% confidence level that HaCaT and B16F10 cells exposed to ions generated at the tested conditions do not significantly alter cell viability.

A final set of experiments was performed to elucidate the duration of membrane defects in both B16F10 and HaCaT cells. These experiments exposed suspended cells to plasma for 600 s and followed the uptake of a nucleic acid probe by fluorescence measurements taken at 1 min intervals. It was expected that membrane premeabilization would enable the uptake of molecules by the cells causing an increase in fluorescence. The rate of fluorescence increase should slow and eventually cease with time corresponding to cells regaining their membrane integrity. Fig. 6 shows raw fluorescence measurements obtained over a 20 min acquisition period for plasma treated B16F10 and HaCaT cells.

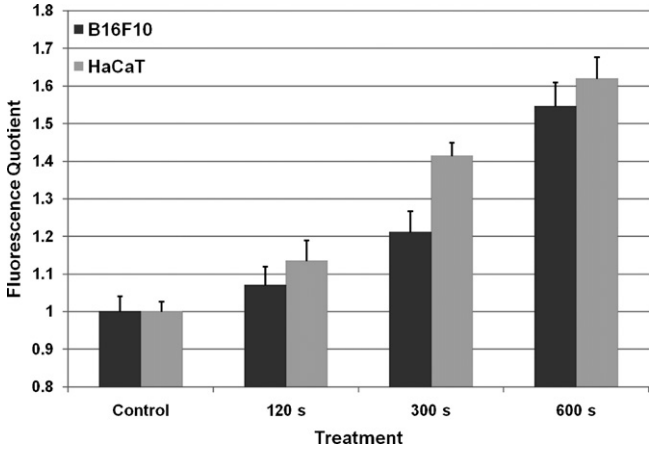

Fig. 4. Fluorescence quotient of cells after plasma mediated delivery of the non-permeant nucleic acid stain Sytox Green. Fluorescence values have been divided by their respective controls (F/F_0) to show a relative increase in delivery. Error bars indicate standard error of the mean.

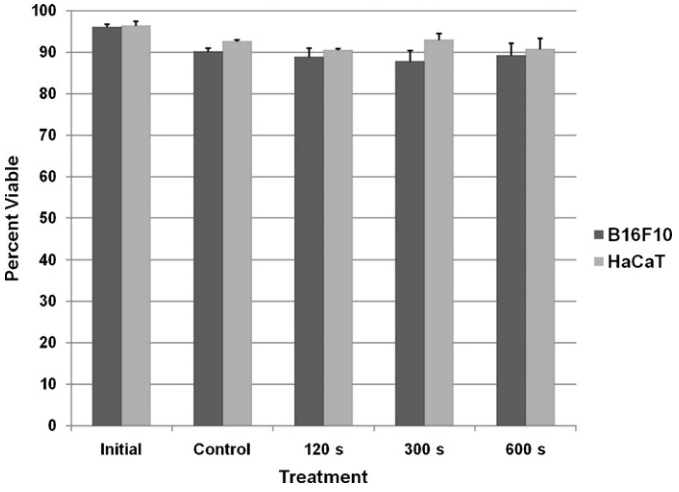

Fig. 5. Viability effects of plasma exposure on cells 4 h after treatment. Error bars indicate the standard error of the mean.

As expected, the rate of Sytox Green influx slowed with time in both cases and changes in the fluorescence levels became statistically insignificant at 6 min for B16F10 cells and 5 min for HaCaT cells. This observation was due to the permeability of the membrane decreasing with time. Differentiating the fluorescence with respect to time allowed the data to be curve fit to determine the rate constant associated with dye exclusion from the cell, which was then correlated to a re-stabilizing cell membrane. A first order kinetic equation, $Y = Y_0 e^{(-kt)}$ provided an excellent fit for the differentiated data with r-squared values of over 0.99 for both HaCaT and B16F10 data. Fig. 7 displays the differentiated fluorescence data (dF/dt) at each observation for B16F10 and HaCaT cells and its associated curve fit. The rate constant, k, found in the HaCaT fit of membrane resealing was 0.28 min^{-1} and the constant for B16F10 membrane resealing was 0.25 min^{-1}. This shows that cells treated with ions to enhance molecular delivery respond similar to cells that have been electroporated (Rols and Teissié, 1990).

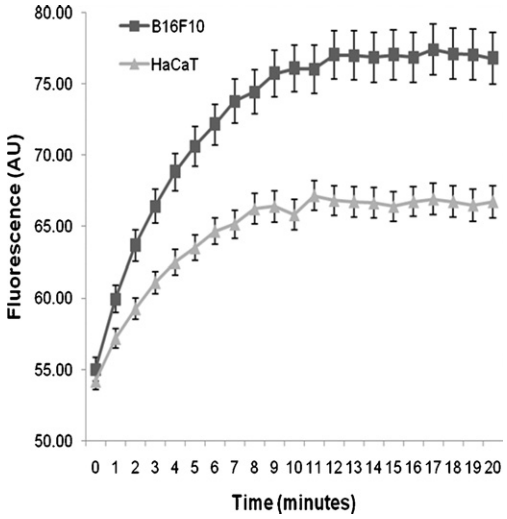

Fig. 6. Raw fluorescence (arbitrary units) measurements of B16F10 and HaCaT cells due to the uptake of Sytox Green after 600 s of ion exposure. Means are plotted at 1 min intervals along with the standard error of the mean.

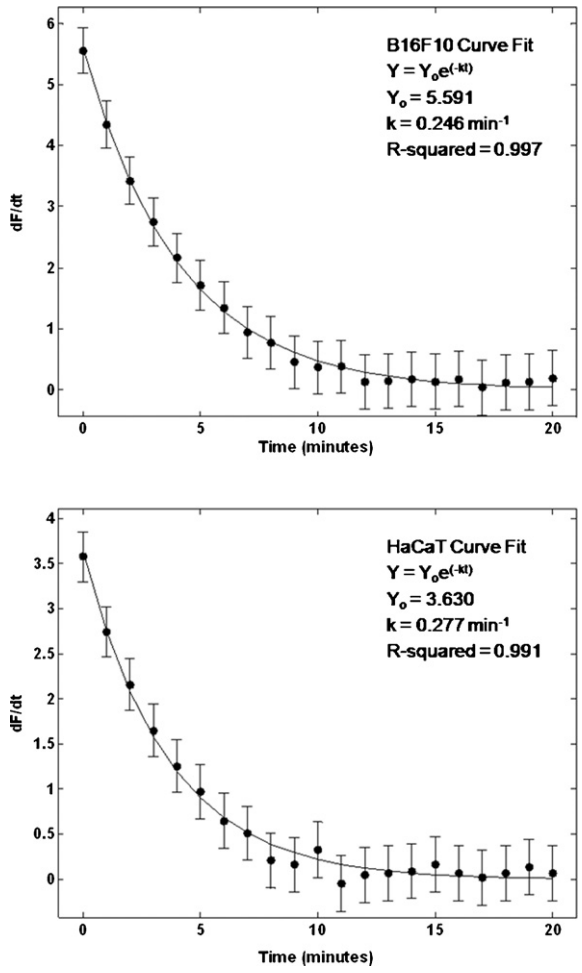

Fig. 7. Plots displaying the change in fluorescence with time for (A) B16F10 and (B) HaCaT cells after 600 s of plasma exposure. Plotted markers indicate the mean and error bars show the standard error of the mean for the data collected. Curves show the fit for a first order kinetic equation.

4. Conclusions

The results obtained using a DC plasma discharge are encouraging with regard to plasma mediated molecular delivery. These experiments show a dose–response relationship between exposure time and the delivery of fluorescent tracer molecules, with significant delivery beginning in both HaCaT and B16F10 cells at 300 s. Maximum delivery of tracer molecules occurred at 600 s of plasma exposure and led to a rise in fluorescence of 62% and 55% for HaCaT and B16F10 cells, respectively. At the tested plasma exposure levels delivery was attainable without harm to the short-term cell viability.

Results from the kinetic experiments offer evidence that plasma exposure and electroporation effect cell membranes similarly. Treatment of cells with either of these modalities causes a temporary increase in the diffusion of exogenous molecules into the cytosol, which is a result of membrane destabilization. In both cases the recovery of membrane integrity and subsequent dye exclusion follows first order kinetics with similar rate constants (0.25 and 0.28 min^{-1}) for the two cell lines tested. These data were consistent with the literature on resealing times for electroporation.

The results from these experiments show this non-contact charge-based delivery system is capable of improving the delivery of small molecules, such as cell tracers or drugs. Coupling this information with other published studies showing the capacity of this system to improve the uptake of plasmid DNA to skin *in vivo* strongly suggests that this novel delivery method is versatile in its ability to locally deliver therapeutic molecules. This work provides evidence that ion deposition causes a transient increase in membrane permeability without the deleterious effects of other field driven modalities, which is a starting place for the discovery of the mechanisms responsible for this phenomena. Further investigation into the effects of applied voltage and exposure time are necessary to begin to elucidate the underlying cause of membrane permeabilization.

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