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Optimization of a Plasma Facilitated DNA Delivery Method

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Abstract

Plasma-based methods have recently emerged as a technique for augmenting plasmid DNA delivery to skin. This delivery modality relies on the deposition of ionized gas molecules on to targeted cells or tissue to establish an electric field. It is hypothesized that this electric field results in the dielectric breakdown of cell membranes, making cells permeable to exogenous molecules. This in vivo investigation sought to optimize the intradermal delivery of a luciferase expressing plasmid DNA by modulating the total exposure to the plasma source and the plasmid DNA dose. Varying the plasma exposure time from 2, 5, 10, and 20 minutes allowed the conditions resulting in the highest expression of luciferase to be found. These conditions correlated to the 10 minute exposure time for a plasma derived from either +8 kV or –8 kV, when the generator was operated 3 cm from the epidermal tissue surface with a helium flow rate of 15 L/min. Exposing the injected flank skin for 10 minutes resulted in a rise of 37.3-fold for a plasma created with +8 kV and 27.1-fold for a plasma created with –8 kV. When using this treatment time with 50, 100, or 200 µg of a luciferase expressing plasmid, it was found that 100 µg resulted in the highest peak luminescence.

Keywords

Plasma; Plasma Delivery; Plasmid DNA; Molecular Delivery; Electroporation; Skin

1 Introduction

The utility of DNA-based therapies and vaccines continues to be exhibited as advances in the understanding of disease prevention and treatment continue to evolve. Delivery of

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Conflict of Interest

The authors of this manuscript are inventors on U.S. patents (US8455228 and US8348942) pertaining to the biological use of plasma discharges.

genetic material to cells results in the production of proteins that induce a biological, biochemical, or immunological response. Research has shown these responses can be used to treat acquired or inherited diseases, as well as prevent disease through vaccination.

The most developed physical method for gene delivery is electroporation. Electroporation utilizes electrodes placed in direct contact with tissues to impart electrical pulses. These pulses generate an electric field that cause ion movement inside the tissue that disrupt the resting cell membrane potential, causing a transient destabilization [1, 2]. The in vivo feasibility of this technology occurred when plasmids were delivered to cells in the skin and liver of rodent models [3, 4]. Building upon these early successes, electroporation has been used extensively to deliver exogenous molecules to cartilage, blood, muscle, epithelial, and loose connective tissue [5-11]. In addition to these basic tissue types, electroporation has also been applied to tumors in order to enhance the uptake of immunomodulatory genes [12-15].

While electroporation has proven to be a highly effective method for performing molecular delivery, it does have some drawbacks. The most notable being that high-energy pulses required to achieve significant delivery often cause tissue damage and patient discomfort [16-19]. This has led to the development of novel electrode arrays where electrode pairs are spaced much closer than in previous applicators. This configuration allows lower voltages to be applied to generate electric fields similar to those used in traditional electroporation, which results in reduced muscle stimulation and does not effects on the efficacy of DNA delivery [20, 21]. Another change occurring in the field of electroporation is the monitoring of changes in the electrical properties of tissues during electroporation sequences with electrical impedance spectroscopy. This approach would allow just enough energy to be delivered to the tissue to induce membrane destabilization without over exposing the tissue and damaging cell viability [22, 23].

An alternative approach to reducing the undesirable characteristics associated with electroporation, such as patient discomfort and tissue damage, is to decouple the applicator from the treatment site. This can be achieved by depositing ionized gases, or plasma, onto a site where exogenous molecules have been inserted. Ion deposition on a targeted tissue leads to the accumulation of a steady state charge and causes an electric potential to be established across the tissue. It is hypothesized that this method works by achieving a critical potential on the epidermal surface that causes ion movement within the tissue to create a mirror charge. Movement of these ions through the tissue would result in destabilization of cell membranes, similar to electroporation.

The utilization of plasma discharges for augmenting molecular delivery has recently been discussed in the literature. The first evidence that this technique could be used for delivering plasmid DNA was performed with a radio frequency generated plasma discharge. This study exposed primary neuronal cells in vitro to a high frequency discharge for one second and successfully delivered plasmid DNA encoding green fluorescent protein [24]. A study conducted later proved direct current plasma could be utilized to deliver tracer molecules to cell in vitro and genes in vivo. This study ionized the air several millimeters above a treatment site and deposited these charged molecules on the cells or tissues below. This

study provided the initial in vivo evidence that ionized gases are capable of enhancing molecular delivery in vivo [25, 26]. Finally, a non-equilibrium direct current plasma source that operates by exciting a flowing stream of helium has been used in vitro to deliver tracer molecules without effecting cell viability [27]. Additionally, this helium-based plasma has been used in vivo to deliver DNA encoding the luciferase protein and the 120 kilodalton envelop glycoprotein associated with human immunodeficiency virus [28-30].

The focus of this study was to optimize the helium-based plasma delivery system for delivering plasmid DNA to murine skin. To accomplish this, plasmid DNA encoding for luciferase was intradermally injected and treated with the plasma discharge, at a spacing of 3 cm from the tissue, for various exposure times. Once the optimum exposure time was determined, these experiments were repeated to determine the plasmid concentration that exhibited the highest peak expression. These data provide the foundation for conducting future experiments with this plasma discharge source.

2 Materials and Methods

2.1 Plasma Generator

The plasma generator utilized in this study was manufactured from a 15 cm long Teflon rod with a 1.5 cm diameter. A 1 cm hole was machined along the length of the rod to create a passageway through which compressed helium could flow. Two electrodes were positioned at both ends of the 15 cm long Teflon tube. A brass encased 5 mW red laser pointer (Instapark, Santa Fe Springs, CA) was positioned on one end of the tube and connected to ground potential. This device served as the reference ground in the system and enabled accurate targeting of the treatment site. An annular stainless steel electrode with a 3.5 mm internal diameter was positioned at the opposite end of the Teflon tube. This electrode was connected to a high voltage direct current power supply (Spellman, CZE200, New York, NY). A gas stream was plumbed into plasma generator body with a polypropylene hose barb placed just below the laser pointer.

A software interface platform (NI 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. Additionally, this platform allowed real time monitoring and logging of the current and potential leaving the high voltage electrode. To serve as a safeguard, a current limit of 100 μ A was set during treatment. The plasma generator system is shown in Figure 1.

The plasma discharge utilized during treatment was created by applying a direct current potential of +8 kV or -8 kV to the annular electrode while flowing 15 L/min of 99.999% helium (AirGas HE UHP300, Kennesaw, GA) through the generator. During treatment, the plasma generator was operated at a distance of 3 cm from the murine flank skin containing the injected plasmid DNA. To stabilize the plasma plume, a high impedance ring with a 1 cm diameter was made from a loop of 22 gauge tinned copper wire and symmetrically encircled the injection site. This ring was held against the epidermal surface with butterfly closure bandages placed around the circumference. The other end of the wire was connected to ground potential through a 1.5 G Ω resistor. This resistance value was selected to limit the

current returning to ground and stabilized the plasma plume from affects of minor air currents.

2.2 Animal Model

Female C57BL/6J (National Institutes of Health, Bethesda, MD) were obtained at six weeks of age and allowed two weeks to acclimate. All experiments were conducted in accordance with an approved Institutional Animal Care and Use Committee protocol. At the conclusion of this study animals were humanely euthanized.

2.3 Anesthesia

Prior to performing experiments and imaging, the mice were anesthetized with isoflurane. To facilitate this, animals were placed in an induction chamber and sedated with 2% isoflurane in a 1 L/min stream of oxygen. Once under deep sedation, mice were transferred to individual nose cones where a constant supply of 2% isoflurane in oxygen was delivered at a rate of 250 mL/min. To minimize anesthesia-induced heat loss animals were placed on a 32°C warming pad during treatment and imaging.

2.4 Plasma Dose Response Study

The first set of experiments discussed in this manuscript sought to determine the effects of plasma polarity and treatment time on the delivery of plasmid DNA. Prior to treatment, intradermal plasmid injections were performed using a 1 mL syringe with a 30-gauge needle. These injections delivered 100 µg of a luciferase encoding plasmid (Aldevron gWiz-Luc, Fargo, ND) in 50 µL of saline. Each experiment consisted of groups of four animals treated with the following methods: 1) No treatment, not injected with plasmid nor exposed to plasma; 2) Luciferase plasmid injection with no exposure to plasma; 3) Plasmid injection and exposure to positive plasma for 2 minutes; 4) Plasmid injection and exposure to negative plasma for 2 minutes; 5) Plasmid injection and exposure to positive plasma for 5 minutes; 6) Plasmid injection and exposure to negative plasma for 5 minutes; 7) Plasmid injection and exposure to positive plasma for 10 minutes; 8) Plasmid injection and exposure to negative plasma for 10 minutes; 9) Plasmid injection and exposure to positive plasma for 20 minutes; 10) Plasmid injection and exposure to negative plasma for 20 minutes. In total, 12 animals were treated with each of the 10 conditions over three independent experiments.

2.5 Plasmid Dose Response Treatments

The second set of experiments discussed in this manuscript sought to determine the effect of modulating the plasmid dose while keeping the plasma exposure time fixed. In these experiments injections of 50 µg, 100 µg, and 200 µg of a luciferase expressing plasmid (Aldevron gWiz-Luc) were made to determine the consequence of plasmid concentration on plasma mediated molecular delivery. The total intradermal injection volume utilized in these experiments remained 50 µL, which was delivered using a 1 mL syringe and 30-gauge needle. Animals were treated in groups of 4 with each of the following methods: 1) No treatment, not injected with plasmid nor exposed to plasma; 2) 50 µg injection of plasmid with no exposure to plasma; 3) 50 µg injection of plasmid and exposure to positive plasma for 10 minutes; 4) 50 µg injection of plasmid and exposure to negative plasma for 10

minutes; 5) 100 µg injection of plasmid with no exposure to plasma; 6) 100 µg injection of plasmid and exposure to positive plasma for 10 minutes; 7) 100 µg injection of plasmid and exposure to negative plasma for 10 minutes; 8) 200 µg injection of plasmid with no exposure to plasma; 9) 200 µg injection of plasmid and exposure to positive plasma for 10 minutes; 10) 200 µg injection of plasmid and exposure to negative plasma for 10 minutes. In total, 12 animals were treated with each of the 10 conditions over three independent experiments.

2.6 Electroporation

During plasmid-dose response experiments, animals were treated with electroporation at each of the plasmid concentrations listed above to serve as a comparison. Electroporation pulses were applied with a four-plate electrode, shown in Figure 2. This applicator uses two sets of parallel plates 4.4 mm wide with a spacing of 6 mm between electrode pairs. A total of eight pulses were delivered where the field was rotated 90° between pulses from the after the 50 µL injection was made in the dermis. These pulses had an electric field strength of 100 V/cm, with a duration of 150 ms, and 1 s duty cycle, which had been previously optimized for delivery of plasmid DNA [31]. To ensure electrical contact was made with the tissue, the electrode surfaces were coated with conductive gel (Parker Laboratories Spectra 360 Electrode Gel, Hellendoorn, Netherlands) prior to treatment. In total, 3 groups of 4 animals per group were exposed to electroporation. The groups for this section of the experiment were: 1) A 50 µg injection of luciferase plasmid with electroporation; 2) A 100 µg injection of luciferase plasmid with electroporation; 3) A 200 µg injection of luciferase plasmid with electroporation. As with the plasma treatments, 12 animals were treated with each of the 3 electroporation conditions over three independent experiments.

2.7 Luminescence Quantification

Luciferase plasmid expression was determined by intraperitoneal injection of 150 mg of D-luciferin potassium salt (Caliper Life Sciences, Hopkinton, MA) per kg of body weight. After injection, animals remained sedated for 15 minutes before capturing the bioluminescence resulting from the oxidation of luciferin. This reaction was captured with a 10 second exposure in an IVIS 200 (Caliper Life Sciences) live animal imaging system. The photon flux (photons/s-cm²) from each animal was quantified with Living Image software (Caliper Life Sciences). This procedure was performed to gather luminescence data prior to treatment and on days 2, 4, 6, 8, 10, 18, 26, and 30 after treatment. Examples of luminescent images acquired are shown in Figure 3.

2.8 Statistical Analysis

Once all data were gathered, the mean photon flux and standard error of the mean was computed for identically treated groups on each follow-up day. An ANOVA test was performed on these data at the 0.05 significance level. Those days having a p-value less than 0.05 were further investigated with the non-parametric Mann-Whitney test to determine statistical significance.

3 Results and Discussion

Operating at an absolute potential of 8 kV produced a stable and reproducible glow discharge emanating from the high voltage electrode. When this electrode was set at +8 kV and operated in an open environment a visible plume measuring 3.0 cm in length was created. Similarly, setting this electrode to -8 kV produced a glow discharge measuring 2.0 cm in length. All treatments involving the plasma discharge were conducted at a distance of 3 cm from the bolus created by the injection. Average currents of 17.7 μ A and -15.1 μ A were observed when the plasma was operated at +8 kV and -8 kV, respectively. This produced a static potential on the epidermis of +5.6 kV for positive plasma and -5.0 kV for negative plasma.

The goal of the first set of delivery experiments was to determine the treatment time that generated the greatest overall plasmid expression. To perform this, 100 μ g of luciferase expressing plasmid was injected intradermally in a volume of 50 μ L. After injection the sites were treated with either positive or negative polarity plasma for 2, 5, 10, or 20 minutes.

Figure 4 shows observed luminescence levels for all groups treated with either polarity plasma. Data points expressed at day 0 represent luminescence values prior to treatment. In all groups peak expression occurred on day 2 and remained at the same relative level until day 21.

Mean luciferase level comparisons were made on day 2 relative to the response that resulted from injecting animals with the luciferase encoding plasmid only. Data collected from 2 minutes of plasma exposure revealed a rise of 12.9-fold when positive plasma was used to treat the injection site and 20.6-fold when negative plasma was used. Exposure to plasma for 5 minutes resulted in an increase of 13.8-fold for positive and 18.4-fold for negative polarity. Plasma exposure for 10 minutes resulted in increases of 37.3-fold for positive and 27.1-fold for negative polarity. Finally, 20 minutes of plasma exposure increased expression by 16.7-fold and 24.5-fold for positive and negative polarity, respectively. Figure 5 shows the data collected on day 2 to formulate these comparison values.

Analysis of these data, using 0.05 as the level of significance, showed that statistically significant rises existed in all groups exposed to the plasma discharge following plasmid injection when compared to plasmid injection alone. Additional data collected for the 2 minute exposure condition showed significantly higher expression for 7 of the 30 follow-up days. Data from the 5 minute exposures showed significantly higher expression for 21 days. Animals exposed to 10 minutes of plasma retained significantly higher expression for 14 days. Finally animals exposed to 20 minutes of plasma following injection retained higher expression for 21 days.

The goal of the second set of delivery experiments was to determine if a dose-response relationship could be established between the plasmid dose and the amount of expression generated with the most effective plasma treatment time. Performing these experiments involved intradermally injecting 50, 100, or 200 μ g of plasmid DNA, suspended in 50 μ L of saline, and treating the bolus with the 10 minutes of positive or negative polarity plasma. As a basis for comparison electroporation was performed with the same plasmid doses using a

four-plate electrode. The selected electroporation signature imparted 8 pulses at a field strength of 100 V/cm with a duration of 150 ms each. Mean luminescence values are shown in Figure 6 for all groups treated. Data points shown at day 0 represent luminescence values prior to performing treatments. As in the first experiments, all groups exhibited their peak luciferase expression on day 2.

Mean luminescence levels were compared on day 2 relative to those animals receiving injection of luciferase encoding plasmid only. Data collected from animals injected with 50 μ g of plasmid and treated with positive plasma had an increase of 16.4-fold, negative plasma increased luminescence by 19.4-fold, and electroporation increased luminescence by 25.5-fold. Animals injected with 100 μ g of plasmid and treated with positive plasma had an increase of 38.4-fold, negative plasma increased luminescence by 27.6-fold, and electroporation increased luminescence by 42.3-fold. Finally, animals injected with 200 μ g of plasmid and treated with positive plasma had an increase of 32.0-fold, negative plasma increased luminescence by 19.1-fold, and electroporation increased luminescence by 66.5-fold.

Figure 7 shows the data collected on day 2 to formulate these comparison values.

Analysis of these data showed statistically significant rises over injection alone for each of the treatments carried out following the administration of the plasmid. Additionally, data collected for the 50 μ g plasmid dose showed significantly higher expression in the plasma treated and electroporated groups for all 30 of the follow-up days. In this group no statistical difference was present between those treated with positive plasma, negative plasma or electroporation. Data collected for the 100 μ g plasmid dose showed significantly higher expression in both plasma treated groups for 14 days and all 30 days retained statistical significance for electroporation treated groups. At this level, no statistical differences were present between those treated with positive plasma, negative plasma, or electroporation for the first 14 days. However, on days 21 and 30 the animals treated with electroporation had significantly higher means than animals treated with the plasma discharge. Data collected from the highest dose, 200 μ g, showed significantly higher expression in the plasma treated and electroporated groups for 14 days compared to injection alone. This dose level showed no statistical differences between those treated with positive plasma, negative plasma, or electroporation.

4 Conclusions

This study has demonstrated the deposition of ionized gases on the epidermal surface of the skin is capable of augmenting plasmid DNA delivery to the dermis. Furthermore, a dose-response relationship is evident when the plasma exposure time or plasmid concentration is changed. The results obtained by increasing the exposure time indicated luminescence rises with increased treatment times. The first set of experiments showed that the maximum luminescence occurs within 10 minutes of plasma exposure for either polarity, when the generator is spaced 3 cm from the tissue surface. Exposing the injected flank skin for this amount of time correlated with a rise of 37.3-fold for positive and 27.1-fold for negative plasma, when compared to animals that had only been injected with plasmid. Exposing the

tissue beyond 10 minutes does not appear to increase expression, as animals exposed to 20 minutes of plasma had a decrease in luminescence compared to those exposed for 10 minutes. This decrease is likely due to a change in cell viability due to the extended presence of membrane defects.

Proceeding with the optimum 10 minute treatment time from the first set of experiments, the effects of modulating the DNA dose also gave surprising results. As the dose was increased from 50 to 100 μg , a statistically significant increase in luminescence was seen in all groups. However, as the dose was raised from 100 to 200 μg , the effects became insignificant in the plasma treatment groups. This allowed an optimum DNA dose to be determined for this system. The 100 μg dose, coupled with 10 minutes of plasma exposure, gave similar results to those found with electroporation.

Further elucidating the mechanism responsible for this delivery modality will be required in moving this technique forward. This will involve determining the effects of operating the generator at higher potentials or distances closer to the tissue, as this will increase the localized field effect and potentially reduce treatment times. Also histological studies will be required to determine the impact non-thermal plasma exposure has on tissues exposed to the discharge and which cells are transfected with plasmid DNA.

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Highlights

- Deposition of ionized gases on epidermal surface of skin is capable of augmenting uptake of intradermally injected plasmid DNA.
- A dose-response relationship between plasma exposure time and plasmid DNA expression is observed in the bioluminescence data. Groups treated with 10 minutes of plasma discharge after injection has the highest luminescence.
- Modulating the DNA dose from 50 to 100 μg caused a significant increase in luminescence in all groups. Raising the dose from 100 to 200 μg caused the effects became insignificant in the plasma treatment groups.
- Results obtained with the plasma-based delivery system were similar to those obtained with electroporation.

Figure 1.

A) Plasma generator mounted on a micromanipulator and connected to an enclosure containing the high-voltage low-current power supply, rotameter for controlling helium flow rate, and National Instruments data acquisition and control hardware. The power supply and data acquisition hardware are controlled using a National Instruments LabView program. B) Plasma discharge captured with a 60 s exposure while flowing 15 L/min of helium and operating the high voltage electrode at +8 kV.

Figure 2.
Four plate electroporation applicator.

Figure 3.

Representative images of animals overlaid with luminescent flux. Images were acquired on day 2 for animals that received (A) no treatment, (B) injection of plasmid only, (C) plasmid with 10 minute positive plasma treatment, and (D) plasmid with 10 minute positive plasma treatment.

Figure 4. Luminescence data for animals exposed to various plasma treatment times. Mean photon flux (photons/s-cm²) and standard error of the mean are shown for animals that received (■) no treatment, (○) injection of plasmid only, (▲) plasmid with positive plasma treatment, and (▼) plasmid with negative plasma treatment. Data represented in plot A corresponds to animals exposed to plasma for 2 minutes, plot B corresponds to 5 minute exposures, plot C corresponds to 10 minute exposures, and plot D corresponds to 20 minute exposures. Each of these groups consisted of 12 animals that were treated 4 at a time in 3 separate, but identical experiments.

Figure 5.

Peak luminescence for animals exposed to various plasma treatment times. Luminescence is represented by photon flux (photons/s•cm²) observed on day 2 for animals exposed to either (A) negative or (B) positive polarity plasma. Error bars represent standard error of the mean. A break in the y-axis was provided to allow visualization of the plasmid only group.

Figure 6. Luminescence data collected for animals injected with various concentrations of plasmid and treated with 10 minutes of plasma. Mean photon flux (photons/s·cm²) and standard error of the mean are shown for animals that received (■) no treatment, (○) injection of plasmid only, (▲) plasmid with positive plasma treatment, (▼) plasmid with negative plasma treatment, and (◆) plasmid with electroporation. Data represented in plot A corresponds to animals that received 50 µg of plasmid, plot B corresponds to 100 µg injections and plot C

corresponds to 200 μg injections. Each of these groups consisted of 12 animals that were treated 4 at a time in 3 separate, but identical experiments.

Figure 7. Peak luminescence for animals injected with various concentrations of plasmid and treated with 10 minutes of plasma. Luminescence is represented by photon flux (photons/s•cm²) observed on day 2 for animals injected with 50 µg, 100 µg, or 200 µg of plasmid prior to exposure to positive plasma, negative plasma, or electroporation. Error bars represent standard error of the mean.