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The Combined Effect of Heat and Corona Charge on Molecular Delivery to a T-Cell Line *In-vitro*

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by

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of the requirements for the degree of
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Dedication

Thank you to my parents for the never-ending love and support. Dr. Mark Jaroszeski for his guidance in the lab. Dr. Sandra Pettit for pushing me towards great opportunities. Jenna Newman for being there for me when things got tough.

This is for Joe, who is always my inspiration to keep fighting until there is a cure.

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Abstract

Gene therapy and immunotherapy are new up and coming fields in cancer treatment. In August of 2017, the first gene therapy was FDA approved called CAR-T cell therapy("FDA approval brings first gene therapy to the United States," 2017). This therapy uses the patient's own T-cells that are genetically modified to attack the cancerous cells. Currently, it takes 4-6 weeks to genetically modify these T-cells, which can be the difference between life and death for a cancer patient (Cheng, Jun, Jiang, & Xi, 2017). There is evidence that gene electrotransfer can help expedite the genetic modification process by having an easy to use device within the hospital. This could prevent the cells from having to be sent to a specialty lab, as is currently done, and would save shipping time. Traditionally, electroporation was a method that was considered for investigation; however, the main drawback is the need for an electrode to be in contact with the cells they are affecting. This led to the idea of using a newer electrogenetransfer method that involved using corona charge. Corona charge, also known as atmospheric plasma, has been defined as the gathering of charged particles in a neutral fluid (Chelsea M. Edelblute, Heller, Malik, & Heller, 2015; Ramachandran, Jaroszeski, & Hoff*, 2008). The advantage of corona charge is that it does not require electrodes to directly contact cells in order to have an electrical effect.

In this research, the use of corona charge was investigated to determine if it caused an increase in molecular delivery across the membrane in a T-cell line compared to no treatment. The addition of heat at elevated temperatures 37°C, 40°C, and 43°C was determined if they caused a statistical increase alone and in combination with corona charge. Also, cell viability was

also tested because the cells needed to maintain high cell viability to be used in treatment. T-tests were done to determine if the difference between treated and untreated cells was statistically significant. It was shown that treating the cells with corona charge for 3 minutes at 10kV and 25μA caused a statically significant increase in molecular delivery while maintaining high viability. Heat did not cause a statistically significant effect on molecular delivery. Combined corona charge treatment and heating to 37° and 40° C resulted in a statistically significant increase in molecular delivery compared to controls that were only heated. Additionally, combined corona charged treatment and heating to 40°C when compared to a control at room temperature, showed a statistically significant increase in molecular delivery in comparison to a sample that underwent corona charged treatment at room temperature.

Chapter 1 Motivation

In August 2017, the Food and Drug Administration (FDA) approved a treatment known as Chimeric Antigen Receptor T-cell Therapy (CAR-T cell therapy). It was approved to treat people under the age of 25 with B-cell leukemia. This therapy takes T-cells from either the patient or the patient's bone marrow donor and genetically modifies the cells to attach to the protein CD19, a protein that is found in all stages of B-cell growth. Once the T-cell attaches to the B-cell, it destroys the B-cell, thus eliminating the cancer and all possibilities of relapsing ("FDA approval brings first gene therapy to the United States," 2017).

Currently, it takes about three days to extract T-cells from the patient. From there, it takes four to six weeks to genetically modify the T-cells and to grow them to sufficient numbers for use. They are sent away to a very specialized lab to be modified and grown; thus, the total time for extraction, modification, and production of enough cells is about four to six weeks. It is possible with newer technologies to shorten that time to allow the therapy to be administered to the patient in a shorter period of time, which could be beneficial ("CAR T-Cell Therapies," 2015). The main motivation for this research is to reduce the time it takes to create transfected T-cells in sufficient numbers by developing an efficient transfection device and method that could potentially be used in any clinical lab to make modified T-cells. This could eliminate the need for long processing as well as shipping times. This study was designed to investigate the use of corona charge and possibly a combination of corona charge and elevated temperature as a method for delivering tracer molecules to T-cells using a form of electrogenetranser. Electrogenetransfer is a more reliable way of genetically modifying cells than other methods like

heat shock and does not require materials like viruses for the cell to uptake the new DNA. This device would allow the genetic modification to occur in minutes, followed by a few days for the cells to replicate. This could significantly lower the time between extraction and infusion to a week or two rather than four to six weeks.

Chapter 2 Introduction

2.1 Gene Therapy

According to the National Institute of Health (NIH), gene therapy is a method of using genes to prevent or treat disease. At the moment, it is mostly experimental and currently only used for diseases that have no known cure. Gene therapy can potentially be used to replace a mutated gene that causes disease with a healthy gene, inactivate or knock out a mutated gene, or introduce a new gene ("What is gene therapy?," 2019). On June 25, 2019, clinicaltrials.gov listed approximately 4,000 clinical trials involving gene therapy that were active, recruiting patients, or completed. Over 2,700 of these trials involve treating cancer, including breast, brain, prostate, lymphoma, and leukemia. In May 2019, there were only 17 Food and Drug Administration (FDA) approved gene and cell therapies. Only two of these, Kymriah™ and Yescarta®, are gene therapies specifically for cancer treatment ("Approved Cellular and Gene Therapy Products," 2019). Both of these drugs use a system called CAR-T cell therapy.

2.1.1 CAR-T Cell Therapy

Chimeric Antigen Receptor T-cell therapy is also known as CAR-T cell therapy. This therapy uses CARs, genetically engineered receptors inserted into T-cells, to target and destroy tumor cells. This therapy uses the patient's own T-cells or the cells from their bone marrow donor (Cheng et al., 2017). In this therapy, T-cells are extracted from the patient, genetically modified with a CAR, and then new cells are inserted back into the patient. Kymriah™ was the first FDA approved gene therapy for cancer treatment being approved in August 2017. CAR-T cell therapy is currently only FDA approved to treat B-cell precursor acute lymphoblastic

leukemia (ALL) in patients under the age of 25 and for adults with relapsed or refractory large B-cell lymphoma ("FDA approval brings first gene therapy to the United States," 2017).

CAR-T cell therapy paved the way for a new wave in treating cancer. This method allows for targeted treatment of leukemia, which has not been done before. For B-cell ALL, the current treatment plan is three years of chemotherapy. While CAR-T cell is currently used for patients who have relapsed, eventually, it could become the front line of defense when it comes to treating childhood leukemia. Both Kymriah™ and Yescarta® target B-cells, so they target a surface protein found in B-cells through all stages of development called CD19. They are designed for B-cell leukemias and lymphomas, and there currently is no way to target specifically unhealthy B-cells so it attacks all B-cells, getting rid of the cancer and even preventing the cancer from returning. While this does mean that the patient will need immunoglobin shots for the rest of their lives, it can be better than some of the side effects from chemotherapy, including chemo brain, infertility, and higher chances of other cancers developing in the future ("Important Facts," 2018).

Treatment cost and CAR-T cell production time are two major obstacles that potentially prevent the widespread use of the therapy. When it was first approved by the FDA, one round of Kymriah™ cost $475,000. As of January 2019, the cost has gone down to $373,000 (Paton, 2018). Thus, it is expensive. Time is also a big issue because it currently takes 4-6 weeks to genetically modify and grow the T-cells before they're introduced back into the patient. Two factors that are effecting this are shipping and limited facilities. Currently, after the cells are extracted, they are sent off to one of the very few specialty labs. In Florida, there are only 4 Kymriah™ treatment centers, and only 95 in the entire country where the cells can be collected from patients before being sent to the specialty labs via courier. Because of this, there is a lot of

time spent transporting the cells. Patients also have to spend time and money traveling to a facility to offers the therapy. This can often mean being away from their friends and family and adding to their treatment costs.

A rapid and efficient transfection method (the introduction of new genes by a non-viral method) that can be performed in a hospital, rather than in a highly specialized facility, could improve on the two obstacles mentioned above. This could result in a higher fraction of the patient's extracted T-cells being genetically modified. This would save cell processing time and cost. If the method were simple enough, it could be done locally in a hospital instead of at a specialized lab. This could save costs as well as any complications or time from transportation. The way to achieve this is by looking at the method of gene delivery. This study focused on using electrogenetherapy, specifically using corona charge.

2.2 Methods of Gene Delivery

There are two main classes of gene delivery; they are, viral and non-virial systems. Non-viral methods can have a physical or chemical basis (Cheng et al., 2017). Both methods have their advantages and disadvantages.

2.2.1 Viral Delivery Methods

Viral delivery is one of the more common forms of gene delivery because of its high transfer efficiency (R. J. Connolly, Lopez, Hoff, & Jaroszeski, 2009). There are a plethora of different viruses that can result in different expression characteristics. Viruses are a common way of genetically modifying T-cells for CAR-T cell therapy. The viral vectors that are commonly used are retroviruses, adenoviruses, and adeno-associated viruses. The most popular are genetically engineered retroviruses (Cheng et al., 2017). Retroviruses are known for their stable replication but only affect duplicating cells. While viruses have high transfer efficiency, meaning

the clinically necessary number of T-cells can be grown in a short amount of time, there are drawbacks. They pose a safety hazard because there is always the danger that the virus will mutate. This could induce an immunoreaction that could lead to tumorigenesis and toxicity (Guo, Li, Bartlett, Yang, & Fang, 2008). While transfer efficiency is an appealing trait, the possibility of mutation and toxicity is a real problem when working with cancer treatments.

2.2.2 Non-viral Delivery Methods

Non-viral delivery methods can be split into two main categories: chemical and physical. Chemical delivery methods include the use of liposomes. Liposomes are molecules comprised of lipid molecules forming a vesicular structure. Liposomes can encompass the desired molecules, genes, or DNA segments. Their dynamic properties allow them to be more customizable. There are many shapes, sizes, and polarities that can be formed by liposomes to ensure the DNA or genes are delivered correctly to the correct cells (Balazs & Godbey, 2011). Liposomes are mostly made up of lipids, meaning they are more biocompatible than other forms of delivery. They also have a low immunogenic response, especially compared to viruses. Unfortunately, liposomes also have their drawbacks. This includes instability, the liposomes getting filtered out of the body before they can affect the cells, the possibility of toxicity (Barba, Bochicchio, Dalmoro, & Lamberti, 2019), and lower expression levels (Heller, Shirley, Guo, Donate, & Heller, 2011).

Physical methods include methods that use physical force to puncture the cell membrane to introduce new genetic material (Du et al., 2018). These methods are becoming more popular in gene delivery because there is nothing that is toxic and/or immunogenic and low cost. Some physical methods are non-invasive or minimally invasive; these include methods such as sonoporation and magnetoporation (Du et al., 2018). Another method is heat shock but it is used mostly in bacteria. It involves the cells and the plasmids being mixed together and set on ice for

a set time. Then the solution is heated to a certain temperature for a short amount of time, then the cells are placed in a growth medium with an antibiotic. The plasmid used normally encodes and antibiotic resistance gene, so only the cells expressing the plasmid grow in the medium. This method works well for bacteria in research because bacteria grow very quickly. It is a low-efficiency method. Thus, it is acceptable of prokaryotes that divide quickly as transfected cells can be cultured rapidly to obtain the desired number of cells. Mammalian cells don't grow as quickly, so the low transfer efficiency is not generally acceptable as it takes too long to grow a sufficient number of transfectants (Sha, Anderson, Liwei, & Haw, 2007).

2.2.3 Electroporation

Electroporation is the formation of small pores (or defects) in the cell membrane when an electric field is applied. These pores can last up to several minutes to allow for molecules, drugs, and DNA fragments to be introduced into the cell (DeBruin & Krassowska, 1999). Irreversible electroporation is a similar treatment procedure that is not used to transport drugs or DNA but is rather used to induce cell death. It uses more severe electrical parameters to do so. Some clinical trials are currently ongoing to kill cancer cells with little to no side effects using irreversible electroporation (Guenther et al., 2019).

If the goal is molecular delivery, then reversible electroporation can be used to deliver either drugs or genetic material into cells. This method can be used for delivering DNA vaccinations, wound healing, and even cancer therapy (Heller et al., 2011). Electroporation is an attractive delivery method because it does not have the same complications as viral or chemical vectors (Ramachandran et al., 2008). It has also been shown to be effective for most cell types with a higher transfer efficiency than other physical methods. Unfortunately, electroporation has resulted in a high cell death rate, which can be useful, but not for gene therapy where the cell has

to live long enough to transcribe the new DNA (Palanker et al., 2006). Furthermore, *in vivo* uses require electrodes have to be in contact with the cells/tissue being treated. This can cause symptoms such as pain, muscle contractions, and damage to the tissue (Ramachandran et al., 2008). This works well for the skin but can become invasive or even impossible for deeper tissues. There is also the issue of patient comfort. If it causes pain, it could turn off some patients from the treatment.

2.3 Corona Charge

A less popular alternative to electroporation is corona charge. Corona charge can be defined as the gathering of charged particles in a neutral fluid (Chelsea M. Edelblute et al., 2015). This has many other names, including atmospheric plasma. There are also many types of corona charge, but the types used in this study are negatively charged corona and non-thermal (cold) produced at atmospheric pressure. Negative corona is created when an electrode is connected to the power supply through the negative terminal (Van Veldhuizen & Rutgers, 2001). Non-thermal plasma occurs when the free electrons are energized by the electrical energy while the rest of the particles stay at room temperature (Chelsea M. Edelblute et al., 2015). It has the advantage of being easily integrated into the currently existing systems and being cost-effective (Sidik et al., 2018).

Corona charge is currently being used for clinical and biological applications. It is being used to inactivate bacteria such as *E. coli* and *S. epidermidis* on different surface configurations (Chelsea M Edelblute, Malik, & Heller, 2015). There is also a potential of corona charge being used in fields such as dermatology and dental care. In dermatology, it can be used as an efficient way to disinfect wounds, a therapy for different skin infections, and for tissue regeneration (Heinlin et al., 2011). In dental care, it can be used to clean the decay in cavities. In addition,

there is promise in using plasma as high-precision removal of cancer or unwanted cells/tissues (Stoffels, Flikweert, Stoffels, & Kroesen, 2002). Finally, there is potential of using corona charge as an alternative to electroporation for drug and gene delivery into cells ((Richard J. Connolly et al., 2012; Richard J. Connolly, Hoff, Gilbert, & Jaroszeski, 2015; R. J. Connolly et al., 2009; Richard J. Connolly, Lopez, Hoff, & Jaroszeski, 2010; Richard J. Connolly et al., 2011; Ramachandran et al., 2008; Shah, Connolly, Chapman, Jaroszeski, & Ugen, 2012).

2.3.1 Electroporation vs. Corona Charge

Electroporation has the advantage of having a high transfer efficiency as well as not having the side effects that biological and chemical vectors have. For electroporation to work, there also needs to be direct contact between the cells/tissue and electrodes. This can have some adverse side effects including muscle spasms, pain, discomfort and inflammation (Shah et al., 2012). One way of circumventing these effects is by using corona charge. While electroporation can be considered invasive, corona charge can transfect cells without direct contact which could make the process more comfortable for patients and can potentially cause less visible scarring (Chelsea M. Edelblute et al., 2015).

2.3.2 Gene Therapy Using Corona Charge

Corona charge is has been hypothesized to cause membrane permeabilization *in vitro,* enough to transfer genetic material, while still maintaining cell viability (Ogawa et al., 2005). One of the first studies used helium plasma, a charge stream in helium that is also nonthermal. It is similar to corona but in a different matrix. Plasma was used to transfect B16.F10 murine melanoma tumors, in the flanks of mice, with an interleukin-28 (pIL-28) expressing DNA plasmid. pIL-28 was delivered to the cells and temporarily hindered the growth of the melanoma tumor but ultimately did not cause tumor regression. This happened when both electroporation

and corona charge were used to deliver the plasmid (Shah et al., 2012). In another study, a helium plasma source was used to deliver DNA to the skin of mice. Expression was as high as 19-fold greater when corona charge was used to delivery DNA as compared to animals that only received DNA injections (R. J. Connolly et al., 2009). There was a study conducted in 2015 using ambient air corona charge to transfect B16.F10 cells with luciferase *in vivo*. This showed that after 72 hours, there was a 5-fold increase in expression levels with no effect on cell viability (Chelsea M. Edelblute et al., 2015).

Two studies, one published in 2008 and another published in 2010, examined the use of corona charge to deliver Sytox™ (a DNA stain) to B16.F10 and/or HaCaT cells *in vitro*. Sytox™ is a molecule that increases fluorescent emission when binds to nucleic acids (such as DNA and RNA), which only happens after the cell membrane has been compromised. It is a quick way to see if the membrane has been compromised enough to uptake new genetic information after being treated. In the 2008 paper, it was found that 40-50% of the area of the cell culture dish containing B16.F10 cells uptook the Sytox™ after 10 minutes of being treated with corona charge. This area was where the treatment was most effective in the center of the total area of the culture dish. It was also observed that the viability was between 96 and 98% twelve hours after treatment (Ramachandran et al., 2008). Table 2.1 is a summary of the results from the paper published in 2010. It shows the mean percent increase in fluorescence when compared to control samples as well as cell viabilities after treatment (Richard J. Connolly et al., 2010).

Table 2.1: Summary of Results from Corona Charge Delivery of SytoxTM (Richard J. Connolly et al., 2010)

	HaCaT Cells		B16.F10 Cells	
Time (seconds)	Increase (%)	Viability (%)	Increase (%)	Viability (%)
120	13	90.5	7	88.9
300	41	93.0	21	87.9
600	62	90.8	55	89.2

2.3.3 Addition of Heat

It is known that temperature increases result in increased cell membrane fluidity. In 2014, a paper showed that reheated samples of HaCaT cells were able to uptake luciferase plasmid during electroporation better at higher temperatures. Temperatures of 45°C, 43°C, and 40°C for up to two minutes were utilized. The voltages 90V, 75V, 60V and 45V were also tested. It was shown that 40°C at 90V, there was a 3.5 fold increase of gene expression from room temperature. Whereas there was only a 2 and 1.5 fold increase for 43°C and 45°C, respectively. This showed that heat had a positive effect on gene uptake (Donate, Burcus, Schoenbach, & Heller, 2015). If heat had a positive impact on electroporation, it could be hypothesized that heat would have a positive impact on cells treated with corona charge as well.

2.4 Research Goals

There is a need for a cost and time-effective way to make CAR-T cells for immunotherapies in an *in vitro* process. Preferably, a process that can be easily conducted and taught so more facilities could house the process and offer CAR-T cell therapy. Patients currently have to spend time and money to travel to one of the few facilities that can collect the cells to then be sent off to a specialty lab. By creating a process that could be implemented in more places, the immunotherapy could become more accessible.

Corona charge provides a non-contact way to accomplish this. The advantages have been discussed for *in vivo*, but for *in vitro,* it can allow for a more aseptic environment because there is no contact with the cells. In addition, if heat can improve the gene uptake achieved by electroporation, then it could increase the gene uptake when corona charge is used as well.

This study will compare the molecular delivery of samples that have been treated with corona charge and those that have not to determine if corona charge has an effect on molecular

delivery. In addition, the effects of elevated temperatures on molecular delivery will also be investigated both by itself and in combination with corona charge. This will determine if heat causes even more of an increase in molecular delivery than corona charge alone.

Chapter 3 Methods

3.1 Cell Culture

This study used Jurkat, Clone E6-1 cells (ATCC TIB-152, American Type Culture Collection, Manassas, VA) which are immortalized human T lymphocyte cells. These cells are commonly used in cancer drug trials as a model human cell line (Abraham & Weiss, 2004). They were cultured in RPMI 1640 1X with L-glutamine (11875093, Gibco, Grand Island, New York). Media was supplemented with 10% (v/v) Fetal Bovine Serum (Corning 35011CV, Corning Cellgro, New York, New York), 1% (v/v) 200mM L-glutamine (25030081, Gibco), and 1% (v/v) penicillin-streptomycin (15140122, Gibco). The cells were seeded in 75cm^2 flasks (Corning 430641, Corning Cellgro) and grown in a standard 37°C incubator that had a humified environment that contained 5% CO_2. All culture flasks were filled with 13 ml of cells/media when cultures were initiated.

Cell cultures were charged with sufficient cells so that they would contain 1.5 million cells per ml when harvested for experimental purposes. Cell enumeration and viability determination are detailed in Section 3.1.1. The number of cells needed to obtain this concentration was based on the time between initially seeding a flask, the desired harvesting time, and the doubling time of the Jurkat cells. The doubling time used was a running average of the doubling time from the previous subculture cycles. The doubling time in hours was calculated by the following equation:

$$Doubling\ Time = \frac{Time * Log(2)}{Log(Final) - Log\ (Initial)}$$

where Time is the amount of time between subculturing in hours, Final is the final concentration of cells per ml in millions, and Initial is the initial concentration of cells per ml in millions. Once the doubling time was calculated, the desired initial concentration was calculated:

$$Desired = 10^{\left[-\left(\frac{Time*Log(2)}{Doubling\ Time}\right)+Log(Desired\ Concentration)\right]}$$

where Time is the time in hours between the current time and the next time the cells will be subcultured. This gave the initial concentration which then determined the volume from the original flask needed to be used for subculture. This volume was calculated with the following equation:

$$Volume = \frac{Total\ Volume * Desired}{Initial}$$

where the Total volume was 13 ml, Desired is what was calculated above, and Initial was the number of cells in millions per ml in the flask. Once the cells were subcultured, they were incubated for either 48 or 72 hours. Cells were subcultured every Monday, Wednesday, and Friday. On Monday and Wednesday, they were subcultured for 48 hours and on Friday, they were subcultured for 72 hours. Every Friday, the cells were placed into a new 75 cm^2 flask.

3.1.1 Cell Enumeration and Viability

Cell suspensions were enumerated for the purposes of subculturing, experimentation, and determining viability. They were counted using a hemocytometer and trypan blue dye solution, 0.4% (15250061, Gibco). For counting, a solution of 50ml of cell solution and 50ml of trypan blue was used. The following equation was applied to determine the number of cells per ml:

$$C_{Cells} = \frac{N_{Live} \cdot 10{,}000 * C_{Trypan}}{N_{Squares}}$$

where C_{Cells} was the final concentration of cells per ml, N_{Live} was the number of live cells, C_{Cells} was the concentration of trypan blue (in this case, 0.5), and $N_{Squares}$ was the number of squares

counted in the hemocytometer under the microscope. Viability was taken by taking the number of live cells over the number of total cells and multiplying by 100%.

3.1.2 Cell Preparation for Experimentation

The volume of cells that was not used to seed a new culture was prepared for experimentation if the viability of the cell culture was greater than or equal to 95%. If so, the cells were centrifuged at 150 RCF at room temperature for 5 minutes in the media. The media was then aspirated and the cells were re-suspended in Dulbecco's phosphate-buffered saline with calcium and magnesium (PBS) (21-030-CV, Mediatech, Inc, Manassas, VA) to wash them. The suspension was centrifuged again at 150 RCF and the wash PBS was aspirated. The cells were washed two more times in this manner. The resulting cell pellet was resuspended in a small volume of PBS. The suspension was enumerated; the volume was then adjusted to create a 2 million cell per ml suspension. The volume of PBS used to dilute the enumerated suspension was found using the following equation:

$$V_{PBS} = \frac{Initial\ amount\ of\ cell\ solution\ in\ ml * Concentration\ of\ cells\ in\ millions/ml}{Desired\ final\ concentration\ in\ cells\ in\ millions/ml}$$

3.1.3 Cell Storage

Jurkat cells were stored long-term as a suspension of cells in PBS with 10% FBS (v/v) and 10% dimethyl sulfoxide (v/v DMSO) (D2650-5X5ML, Sigma-Aldrich VO LLC, St. Louis, MO). Before placing the cells in liquid nitrogen, they were first placed in a -80° freezer overnight. After that, they were stored in the liquid nitrogen tank. When the cells were to be used, they were quickly unfrozen by holding the vial in a gloved fist. Once the solution was defrosted, it was centrifuged to remove as much DMSO as possible. After the pellet was formed, the excess fluid was aspirated. The fluid was replaced with media and placed in a 75 cm^2 flask. Unfrozen cells were subcultured for several passages before using for experimental purposes.

3.2 Fluorescent Tracer Molecular Delivery

Sytox™ Green Nucleic Acid Stain (S7020, Life Technologies, Eugene, Oregon) is a tracer molecule that fluoresces when it comes into contact with nucleic acids. This can only occur if the membrane of the cell is compromised (i.e. the cell is dead or the corona charge somehow permeabilizes a cell membrane to allow the molecule in). Once the molecule comes into contact with the nucleic acid, the fluorescence increases by more than 500 fold (Richard J. Connolly et al., 2010; "SYTOX™ Green Nucleic Acid Stain - 5 mM Solution in DMSO,").

A 250µL aliquot of Sytox™ Green was obtained from the manufacturer as a 5mM solution in DMSO. For experimentation, it was diluted to a 5µM solution in PBS. The concentration was obtained from the literature, where after treating 700 µl of cell suspension (at a concentration of 2 million cells per ml), a 175 µl aliquot of 2µM Sytox™ was added to the suspension to make a 1µM Sytox™ solution in the sample (Richard J. Connolly et al., 2010). Sytox™ was added after corona charge treatment (no more than 5 seconds after treatment ended) and the solution was mixed by gently pipetting. Three representative 150µl aliquots of treated and control samples were then pipetted into a black, polystyrene 96-well microplate (117017023, Corning Incorporated, Corning, NY) as shown in Figure 3.1 where Blank is PBS, Control is the untreated cell solution and Treated is the treated cell solution.

The plate was then placed into the BioTek FLx800 Microplate Fluorescence Reader (BT-FLX800T, BioTek, Winooski, VT) which was connected to the Gen5 2.09 program, an all-in-one microplate reader software (GEN5, BioTek) using a Dell Latitude Laptop (E6400, Dell, Round Rock, TX). The procedure shown in Figure 3.2 was created to track the fluorescence over 10 minutes. Figure 3.3 shows the arrangement of the entire plate, which follows Figure 3.1.

Blank		
Control	Control	Control
Treated	Treated	Treated

Figure 3.1: The arrangement of solutions in the plate before inserting the plate into the microplate reader.

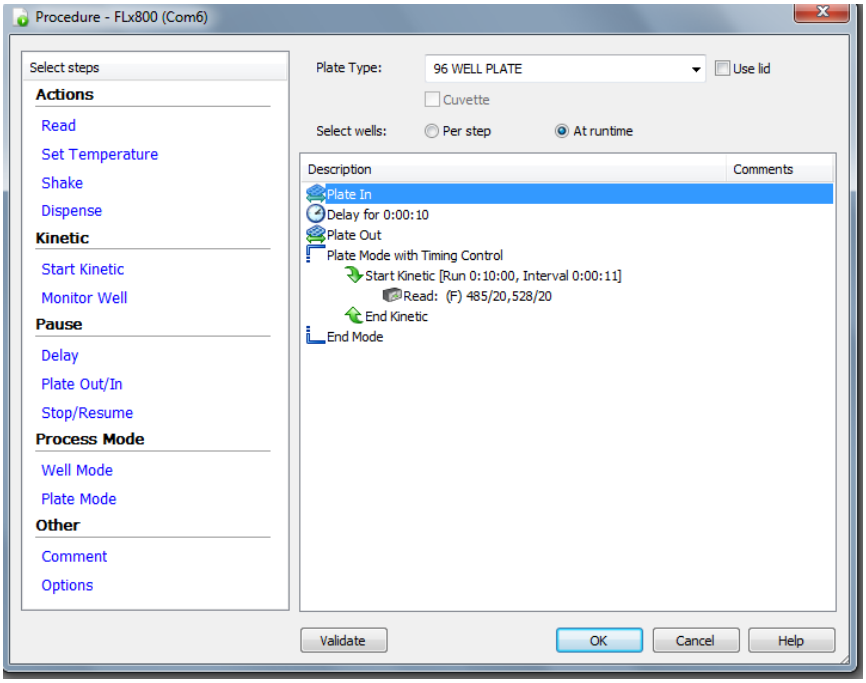
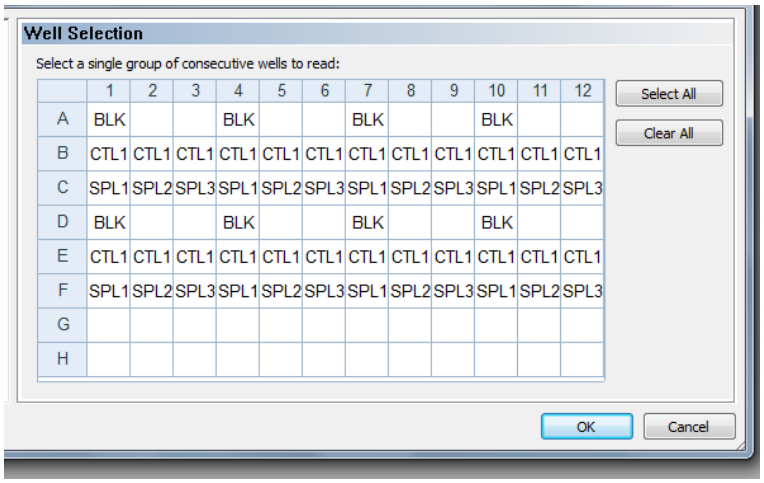


Figure 3.2: The procedure used in Gen5 to quantitate the fluorescence of samples periodically over 10 minutes.

Over the next 10 minutes, the program created the curves of the fluoresce of the control and the treated cells over time by measuring fluorescence every 11 seconds. It was expected that the controls would have less fluorescence than the treated cells. The last point (after 10 minutes) of the three controls were averaged and the last point of the three treated were averaged. The last

point at 10 minutes was used because typically the slope of the curves stops changing before 10 minutes as shown in Figure 3.4. The average of the treated was subtracted from the average of the control and the difference in treated and non-treated was determined. The larger the difference, the more successful the delivery was.



	1	2	3	4	5	6	7	8	9	10	11	12
A	BLK			BLK			BLK			BLK		
B	CTL1	CTL1	CTL1	CTL1	CTL1	CTL1	CTL1	CTL1	CTL1	CTL1	CTL1	CTL1
C	SPL1	SPL2	SPL3	SPL1	SPL2	SPL3	SPL1	SPL2	SPL3	SPL1	SPL2	SPL3
D	BLK			BLK			BLK			BLK		
E	CTL1	CTL1	CTL1	CTL1	CTL1	CTL1	CTL1	CTL1	CTL1	CTL1	CTL1	CTL1
F	SPL1	SPL2	SPL3	SPL1	SPL2	SPL3	SPL1	SPL2	SPL3	SPL1	SPL2	SPL3
G												
H												

Figure 3.3: The microplate configuration, each run has one blank, 3 controls, and 3 samples.

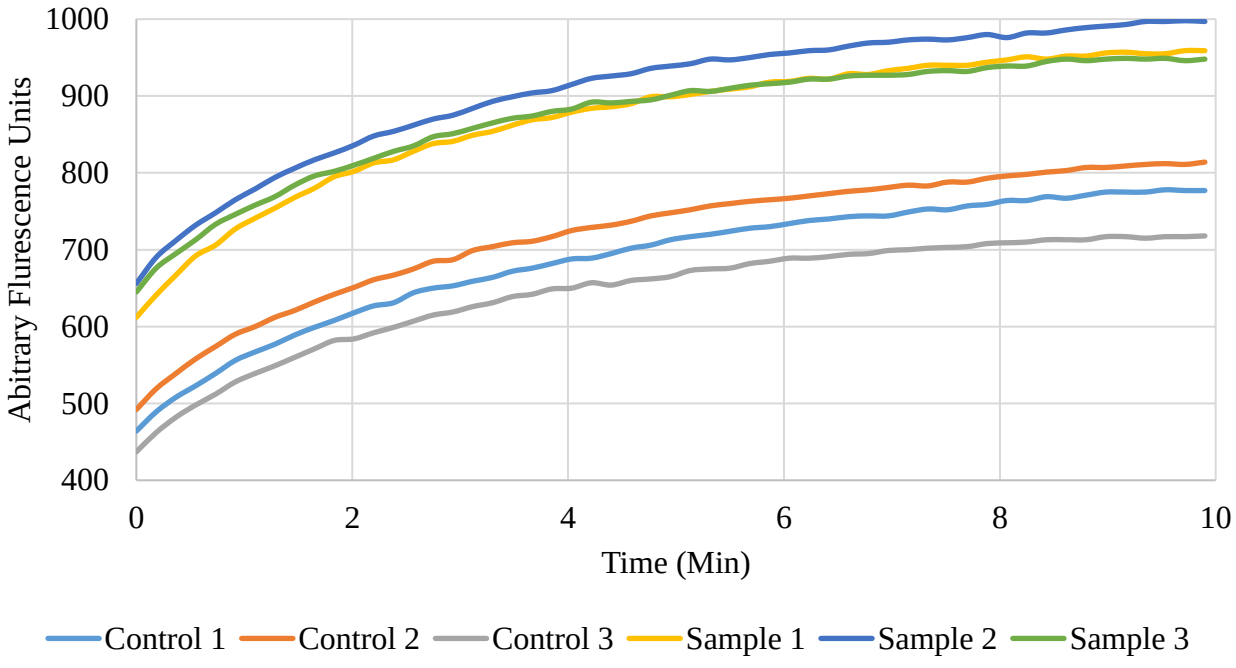


Figure 3.4: Typical curve of cells fluorescence in fluorescence reader after 10 minutes.

3.3 Cell Treatment System

3.3.1 Plasma Generator

The entire treatment system is shown as a schematic in Figure 3.5. All of the components were assembled/connected in order to treat Jurkat cells using a corona charge generator in a modified cell culture dish. The temperature was controlled by a hotplate and temperature was measured using thermocouples. Corona charge was generated by applying a high voltage to the dish using a power supply that was controlled using a custom software, laptop computer, and a digital converting (A/D) card. Voltage and current were monitored using an oscilloscope.

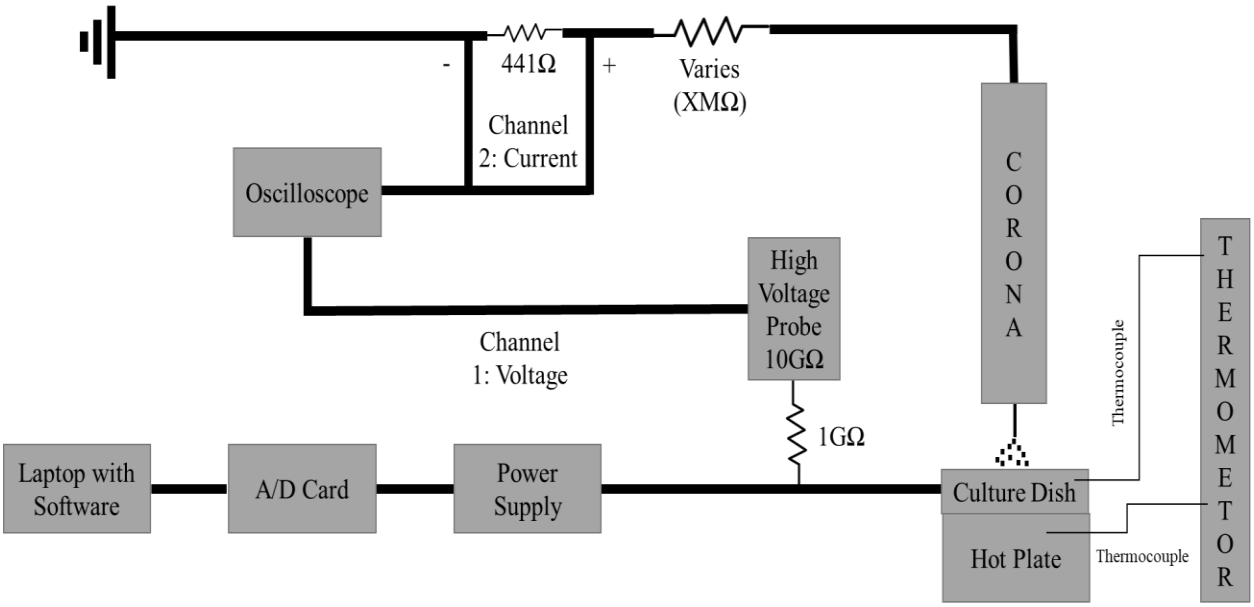


Figure 3.5: Schematic of apparatus used to heat and corona treat cells.

3.3.2 Corona Charge Generator

Plasma was generated using a single needle. The needle was exposed to the ambient atmosphere in a biological safety cabinet where no vacuum or other gases are introduced. The needle used (NA2840, Natural, China) was a 28 gauge needle with a 0.35 mm diameter and a 100 mm length. It was surrounded by a white Delrin tube and the needle protruded approximately 3 mm. Figures 3.6 and 3.7 show the corona generator and needle.

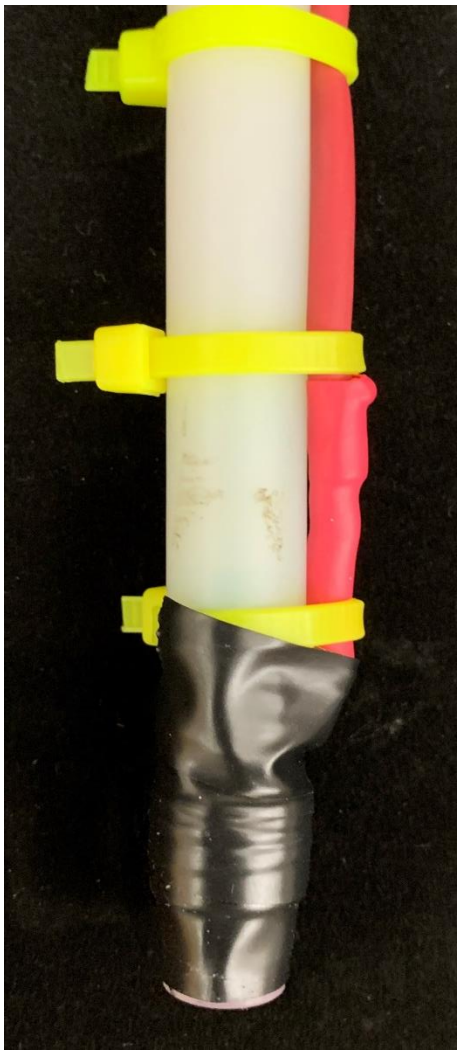


Figure 3.6: Corona generator

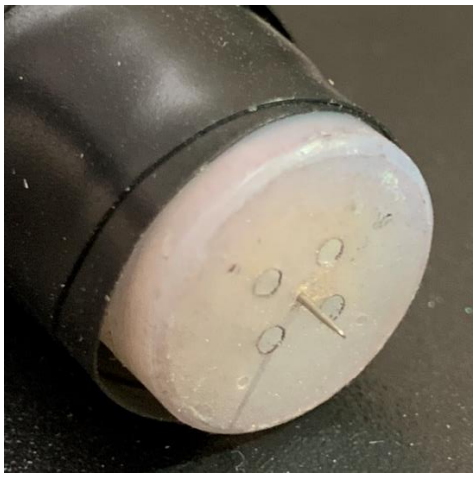


Figure 3.7: Single-needle corona generator

3.3.3 Instrumentation and Control for the Plasma Generator

The hardware used to run and control the plasma generation had two main parts. The first was a data acquisition and control card (DAQ) (182509C-01, National Instruments, Austin, TX) installed in a laptop computer (D630, Dell). The second was a Spellman High Voltage Power Supply (X1778, Spellman High Voltage Electronic Corporation, Hauppauge, NY). The power supply could output a maximum voltage of 30kV and a current of 500μA. If more than 500μA were drawn from the power supply, the output voltage was automatically reduced so that no more than this upper limit of current was output.

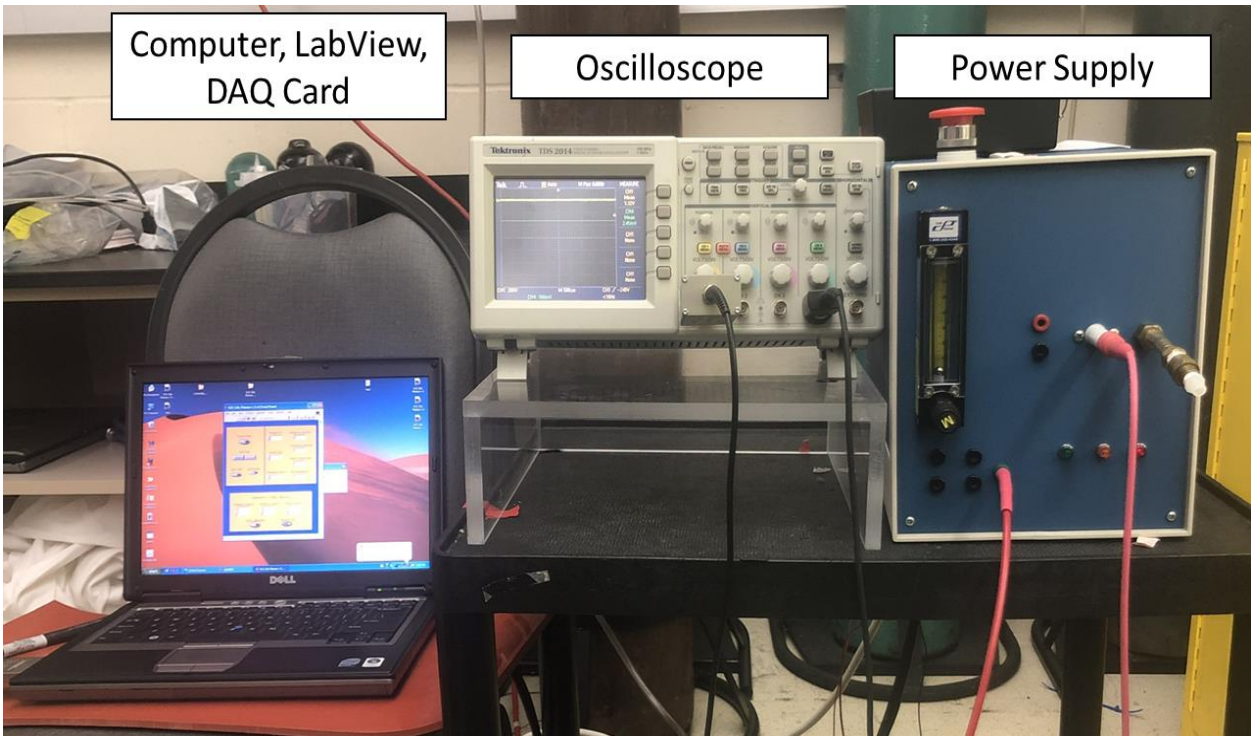


Figure 3.8: Apparatus used to control the plasma generator.

LabView (LabView, National Instruments) software was used to write a custom program and graphical user interface shown in Figure 3.6. This interface allowed the user to input the voltage and maximum current supplied to the coronal generator.

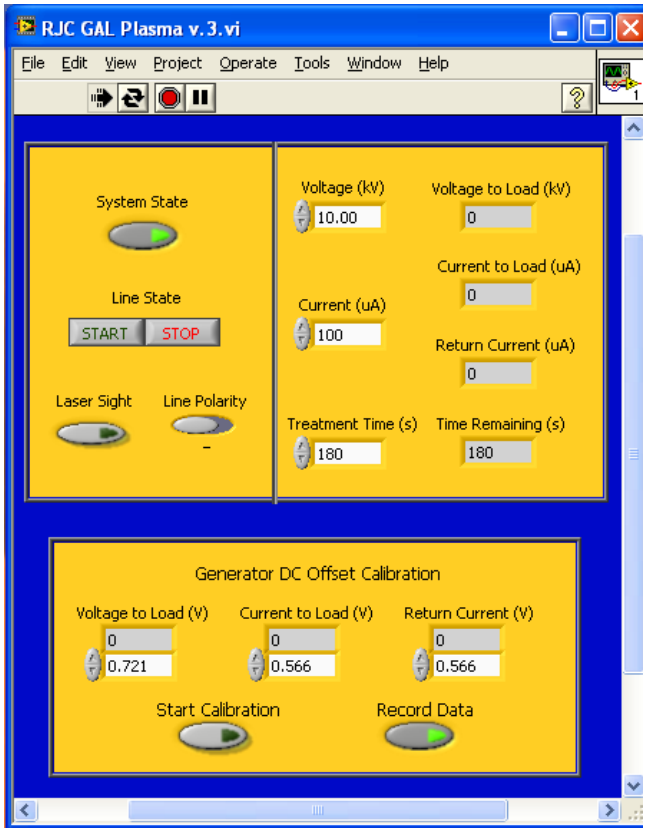


Figure 3.9: User interface displayed on the computer.

The interface also showed the actual current and voltage based upon signals returned from the power supply and digitized by the DAQ. These values were found to sometime be inaccurate. For this reason, a four-channel digital storage oscilloscope (TDS 2014, Tektronix, Beaverton, OR) was used. One channel of the oscilloscope was used to read voltage using a high voltage probe (P6015A, Tektronix) to read the voltage power supply output voltage. This probe was connected across a 1 GΩ resister. A standard 300V (maximum) probe was used with another channel of the oscilloscope. This probe was connected across a small measurement resistor (441Ω) to determine the current. The second channel was used to determine the voltage drop across the resistor and then use Ohms Law, V=IR (Voltage = Current x Resistance), to calculate the current.

3.3.4 Temperature Control Apparatus

One of the challenges with the apparatus was creating a system to heat cells consistently and efficiently. It was decided to use a hotplate (309N0030, Fisher Scientific, Waltham, MA) with an aluminum plate fixed to the top. The plate was used to distribute heat more evenly across the surface. The next challenge was finding a way to determine temperature during testing. Any equipment in or near the cells or dish increases the risk of the generator arcing away from the cells, which would prevent the treatment of the cells. The temperature measuring device had to be accurate enough to differentiate between a fraction of a degree Celsius. It was decided to use two K-type thermocouples (SC-TT-K-30-36-PP, Omega, Norwalk, CT), one that would be taped to the hot plate and one that would be placed into the cell suspension. A thermometer (HH912T, Omega) that had two inputs for thermocouples used with the thermocouple. One thermocouple was placed in the suspension. Thus, it was used to determine when the suspension reached the desired temperature. The other thermocouple was placed on the hotplate to monitor any temperature changes in the hotplate. The reason behind this was that once the cell solution was heated to the correct temperature, as long as the plate did not change more than ±0.5°C, it could then be assumed that the suspension was ±0.5°C from the desired temperature. Figure 3.10 shows the apparatus used to accomplish this. Calibration was essential to ensure the temperature held at the correct temperature without having to test it each time. Inserting the probe each time would compromise sterility and the probe could not be in contact with the solution during treatment. Tests were done with the gold cell culture dishes containing PBS to determine what temperature the hot plate and metal plate should be to obtain the desired temperature. Table 3.1 shows the results of these tests, including the time required to reach the setpoint temperature.

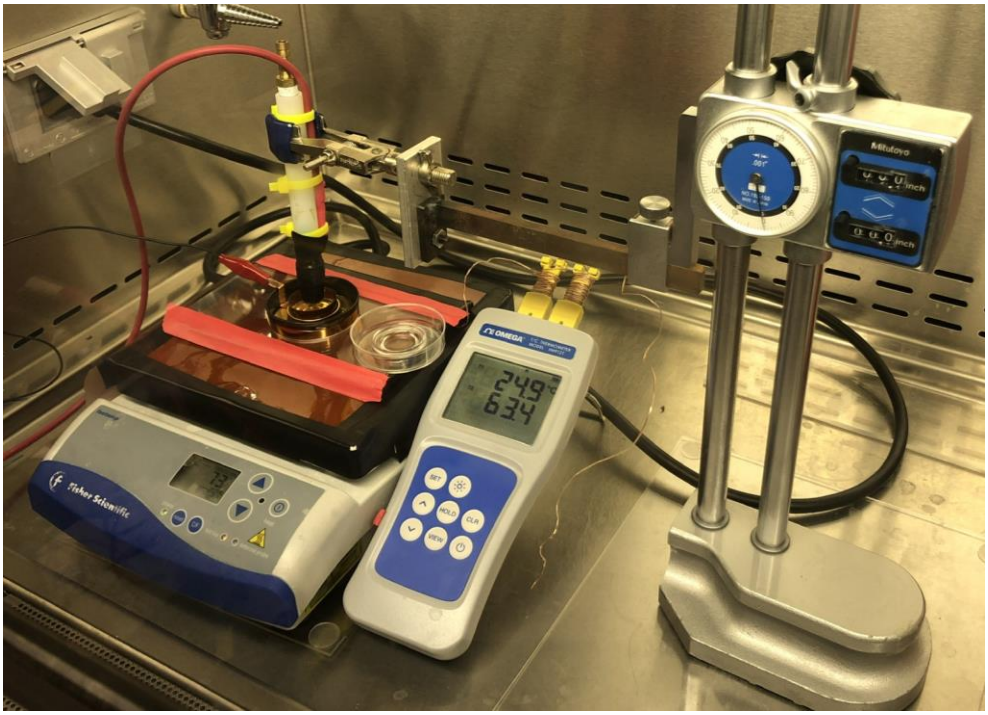


Figure 3.10: Hot plate set up. The thermocouple on the left connects to the metal plate while the thermocouple on the right would be used in the cell solution until corona was applied.

Table 3.1: Hot Plate Temperature Set Up for Experimentation

Temp Desired (°C)	Hot Plate Temp (°C)	Metal Plate Temp (°C)	Ramp Up Time (min)
37	72	62	2.5-3
40	82	68	3
43	85	72	3

3.3.5 Cell Culture Dish Design for Molecular Delivery in Cells

The dishes used were organ culture double-well dishes (Falcon 353037, Corning) that were modified for experimentation. Figure 3.11 and Figure 3.12 show the measurements of the dish.

The dishes were modified to make them able to conduct electricity, gold was sputter-coated onto the dish so that the resulting layer was approximately 100 Angstroms thick. Dishes were first sputter-coated with chrome. The chrome helped keep the gold attached to the dish.

Then, with the exception of the center well where the cell suspension would ultimately be treated, the dish was coated in Matte Clear Enamel (7701830, Rust-Oleum, Vernon Hills, IL). This was to ensure that the corona charge would not arc or stream to not arc to parts of the dish where the cells would not be. Once the enamel was dry, a layer of electrical tape was placed on the inside of the outer well to ensure even if the enamel wore off that there would still be a barrier. Kapton tape was placed around the outside of the outer well for the same purpose. Once the dish was well insulated, a piece of copper tape was placed so that it contacted the uninsulated cell treatment area and formed a pathway to the exterior of the dish. This piece of tape was connected to the high voltage power supply. There was also a 7.5mm tall piece of Kapton that was shaped in a loop to fit in the inner well to ensure that there would not be any arcing to any undesired place on the dish. Figure 3.13 is an image of the dish after it was completed.

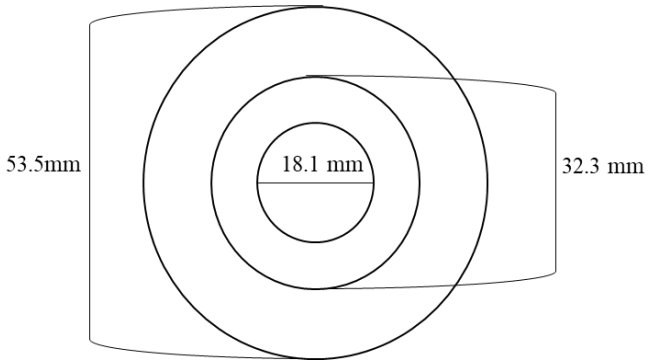


Figure 3.11: Top view measurements of double-well dish used in experimentation.

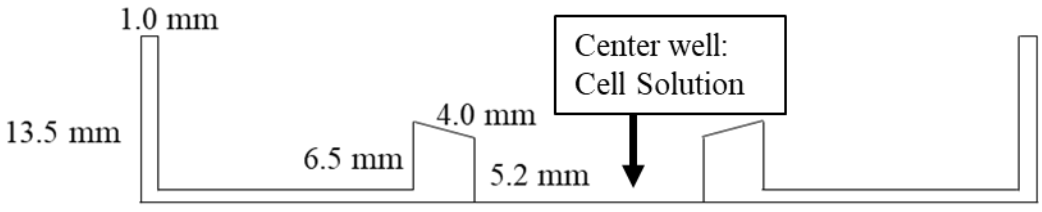


Figure 3.12: Side view measurements of double-well dish used in experiments.

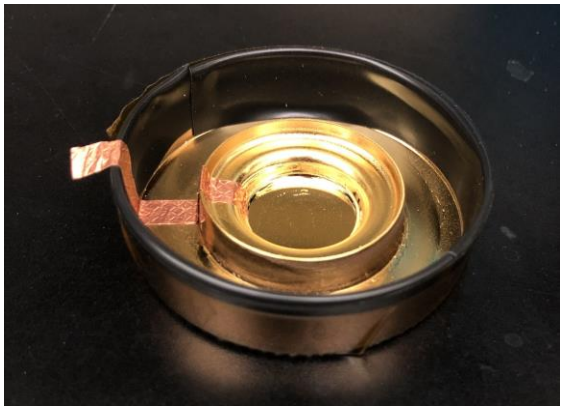


Figure 3.13: Modified cell culture dish used in experimentation.

3.4 Statistical Analysis

All data comparisons were made using a Student's t-Test. The null hypothesis was that the values were not statistically different. All comparisons were made using a level of significance (α) of 0.05 or, a confidence level of 0.95). The following equation was used to compute T values:

$$T_{stat} = \frac{M_1 - M_2}{\sqrt{\left(\frac{1}{N_1} + \frac{1}{N_2}\right)\frac{(N_1 - 1)SD_1^2 + (N_2 - 1)SD_2^2}{N_1 + N_2 - 2}}}$$

where M_1 and M_2 are the mean values of the data sets, N_1 and N_2 are the sizes of the data set, and SD_1 and SD_2 are the respective standard deviations (Duncan, 1983). If the absolute value T_{stat} (calculated T-value) was greater than $T_{critical}$, then the null hypothesis was rejected and there was a statistically significant difference between the two means.

Chapter 4 Results

4.1 Determining Temperature and Corona Charge Treatment Parameters

There are currently no published studies that have used corona charge to treat Jurkat cells, so one part of this first step was to determine treatment conditions for corona charge application that would result in delivery and maintain viability. Similarly, temperatures that might increase membrane fluidity, and perhaps augment corona charge mediated delivery, were unknown. Some initial temperature parameters from published work could be used as a guide. However, these temperatures were used in a different cell line and were combined with electroporation rather than corona charge. All parameters were tested and the results were statistically compared before finalization.

4.1.1 Cell Viability and Permeability Changes of Untreated Control Samples at Ambient Temperature

Experiments were conducted with Jurkat cells in PBS throughout this entire study. In order to determine how long experiments could be conducted using a batch of cells stored in suspension on the benchtop, an experiment was conducted. This experiment examined cell viability and permeability to Sytox^{TM}. Cells were harvested and washed as described above, suspended in PBS to a concentration of 2 million cells/ml, and stored in PBS at room temperature in a 15ml centrifuge tube. This was identical to the conditions the cells would be subject to during molecular delivery experiments. Viability was determined using trypan blue dye and a hemocytometer as described above. Viability was subsequently determined each hour for the next 5 hours. The results are shown in Figure 4.1 and indicated that storing the cells at

room temperature for 5 hours has no effect on cell viability because there was no noticeable decrease. Viability was maintained and was approximately 95% throughout the 5 hour period. It was envisioned that experiments would take less than three hours to complete. Thus, viability changes in a stock suspension of cells stored at room temperature would be negligible.

Sytox™ would be used to determine the effects of heat and/or corona charge on molecular delivery; thus, it was important to determine if the permeability of Jurkat cells changed over a typical experimental time frame. Figure 4.2 shows the fluorescence cell samples that were stored for up to 5 hours at room temperature. Data in the figure was generated by introducing an aliquot of Sytox™ to an aliquot of cell suspension that had been stored for 0, 1, 2, 3, 4, or 5 hours. The final concentration of Sytox™ was 1μM. Within 1 minute of mixing, the Microplate Fluorescence Reader was used to quantitate fluorescence. Figure 4.2 shows that over time, permeability to Sytox™ changed. This is in contrast to viability that did not change over 5 hours. It was determined that including an untreated control sample with each heat and/or corona charge treated sample was absolutely necessary in order to compensate for these changes. This control sample was withdrawn from the stock suspension of cells at the same time as those being treated with heat and/or corona charge. Therefore, the fluorescence of the control could be used to adjust (by subtraction) the fluorescence of the experimental sample.

4.1.2 Cell Viability Changes Due to Heating

The goal of these experiments was to determine if cell viability changes (likely decreases) that result from heating cells to 45°, 43°, 40°, and 37°C for 10, 5, and 2 minutes. These experiments were conducted to define time and temperature combinations that the cells could withstand without significantly reducing viability. It was important to maintain viability

because the ultimate goal was to transfect cells using corona charge at elevated temperatures. Cells must remain alive in order to express the transfected DNA.

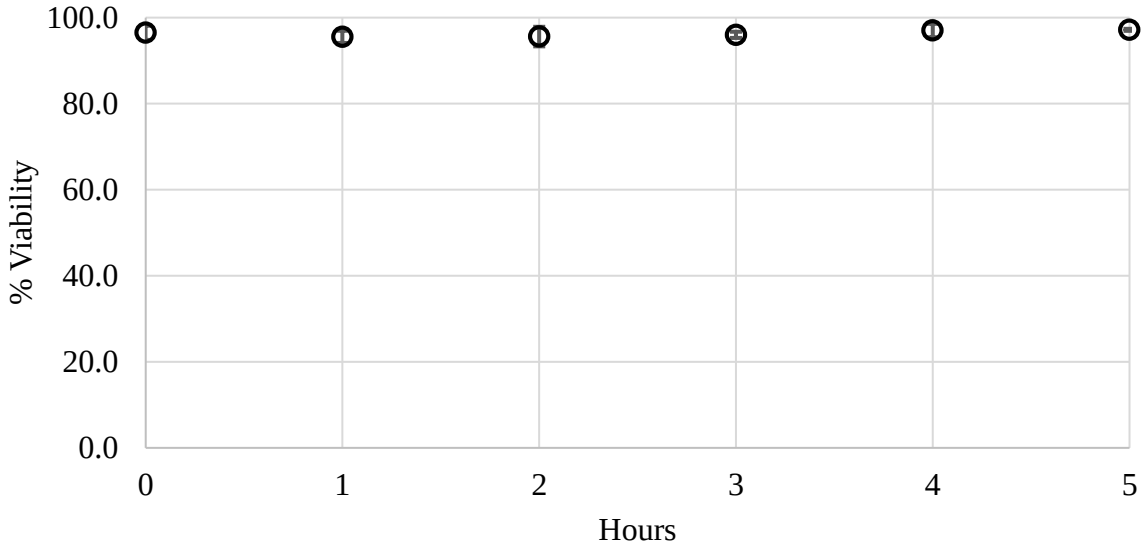


Figure 4.1: Viability of cells stored in PBS for 5 hours at room temperature. N=3 samples

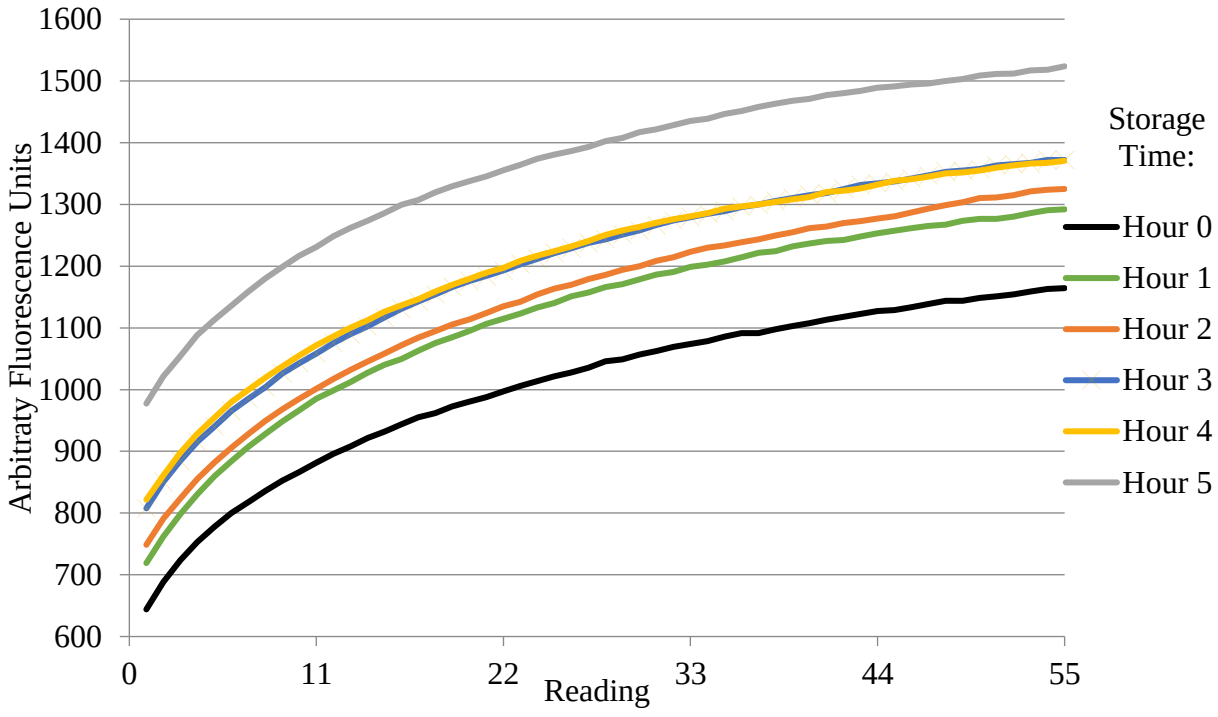


Figure 4.2: Sytox™ uptake for cells stored at room temperature in PBS for five hours.

Temperatures of 45°, 43°, and 40°C were investigated based primarily on a 2015 study (Donate et al., 2015) that heated cells before treating with electroporation. 37°C was also investigated because it is considered human body temperature, and it is also the cell culture incubation temperature. Experiments were conducted by first heating 700µl of cell suspension (2 million cells/ml) in PBS to the correct temperature and then holding the temperature constant for a prescribed time. Cell viability was determined every hour for the first five hours and then again at 24 and 48 hours. Replicate samples were treated the same with respect to heating temperature and time. Temperature hold times of 2, 5, and 10 minutes were used at all temperatures.

Figures 4.3 shows the viability of the unheated cells (those at ambient temperature which was consistently about 25°C) compared to the heated cells at the different temperatures for 10 minutes. Each data point is the mean of a triplicate sample (N=3). For the entirety of the study, the control (unheated cells at ambient temperature) viability average was 95%.

At 43°C and 45°C after being heated for 10 minutes, the viability of the cells immediately after heating dropped to 60-80%. After 24 and 48 hours of incubation, viability dropped dramatically to a range of 5-25%. These were considered substantial adverse effects. In contrast, lower two temperatures (40° and 37°C) showed that viability was reduced by at most a few percents (40°C only) relative to room temperature control cells.

Next, the same temperatures were tested for hold times of 5 and 2 minutes. Figures 4.4 shows viability data for 5 minutes; Figure 4.5 shows similar data for 2 minutes. Data in both figures show that heating the cells at 45°C for 5 and 2 minutes reduced the viability to 31.0% and 39.6% after 24 hours, respectively. After 48 hours, the viability was 27.2% for the cells that were heated for 5 minutes and 42.2% for the cells heated for 2 minutes. While the cells that were heated for 2 minutes had the potential to grow back, the initial viability decreases were high. It

would take too long to grow the cells back to therapeutic numbers. Based on this data, 45°C was determined to cause too much cell death to be used in this study. Anything around 70-80% viability after 24 and/or 48 hours would be deemed acceptable based on Donate's 2015 study (Donate et al., 2015). At 43°C, the viability at 5 minutes was a little low, but acceptable. At 2 minutes the viability changes were approximately within this range. 37° and 40°C showed negligible changes in viability after heating for 10, 5 and 2 minutes.

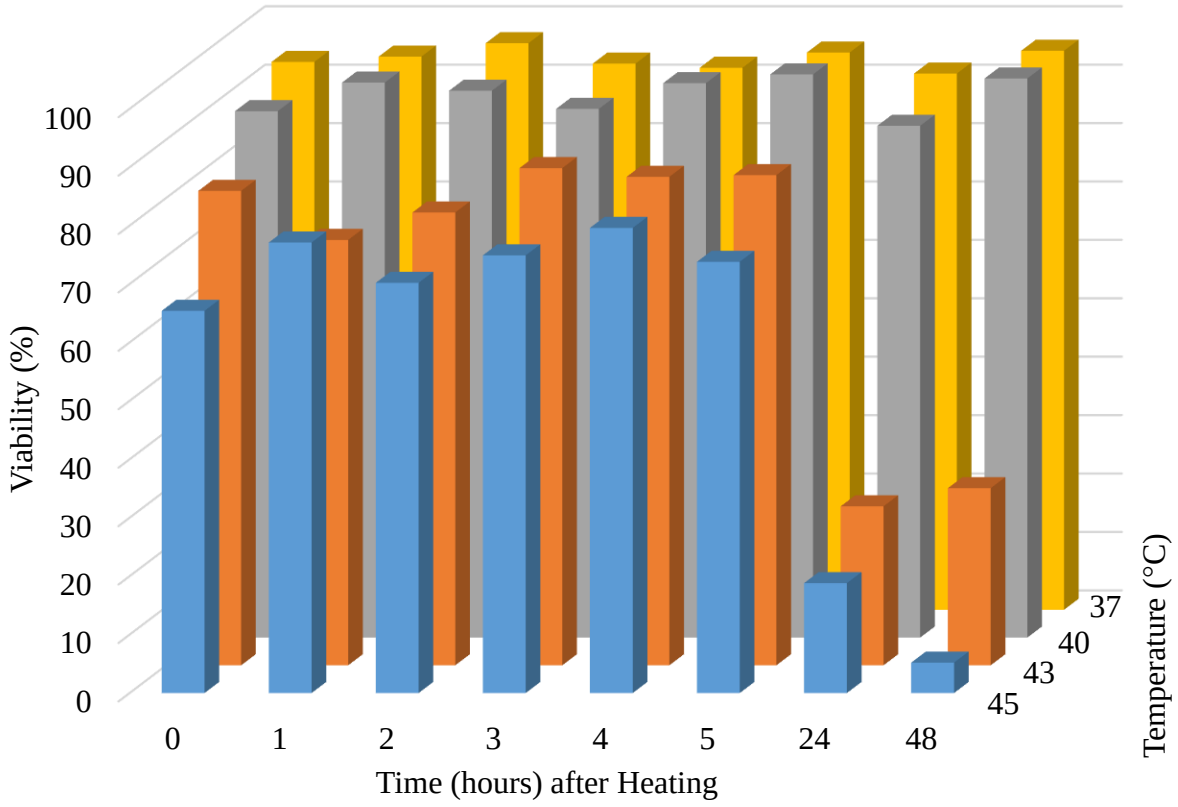


Figure 4.3: Viability of cells after heating to 45°, 43°, 40°, and 37° C and holding temperature for 10 minutes. Viability was determined at hours 0, 1, 2, 3, 4, 5, 24, and 48. N=3 samples per combination of time and temperature.

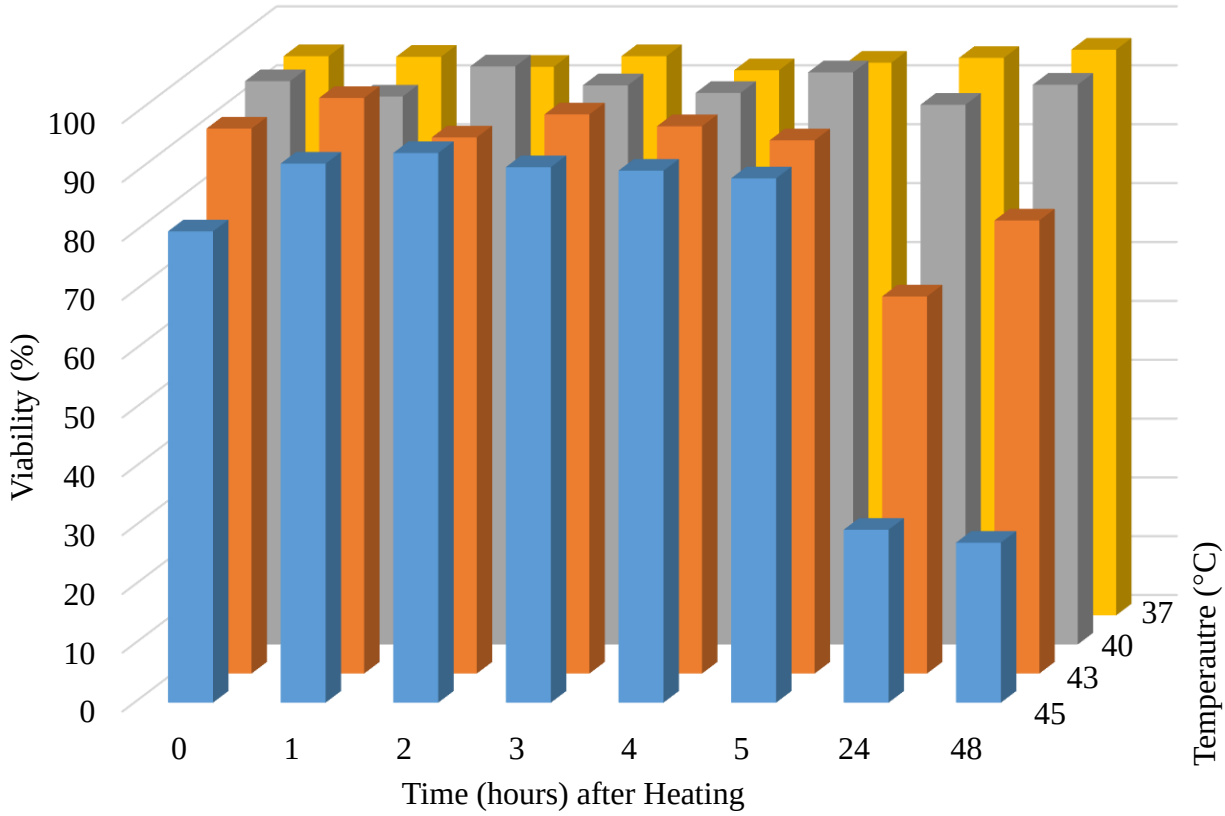


Figure 4.4: Viability of cells after heating to 45°, 43°, 40°, and 37° C and holding temperature for 5 minutes. Viability was determined at hours 0, 1, 2, 3, 4, 5, 24, and 48. N=3 samples per combination of time and temperature.

4.1.3 Cell Viability Changes Due to Corona Treatment at Ambient Temperature

The next step was to determine the viability effects of treating Jurkat cells with corona charge. The goal was to determine parameters that could affect the cells but not affect viability dramatically. Corona charge was generated by applying different negative voltages to the conductive dish using the power supply. Thus, the applied voltage was one parameter used to specify the coronal generating conditions. The needle of the corona generator was then

connected to ground through a high-power resistor which allowed control of the current flowing through the system. Thus, the current was a parameter necessary to specify the corona generating parameters. Treatment time was also a parameter, time that cells were exposed to coronal charge. Voltages used to generate corona charge were 10kV, 12kV, 14kV, and 16kV in this set of experiments. Voltages that were lower than 10kV did not produce a visible plasma plume or result in significant current flow.

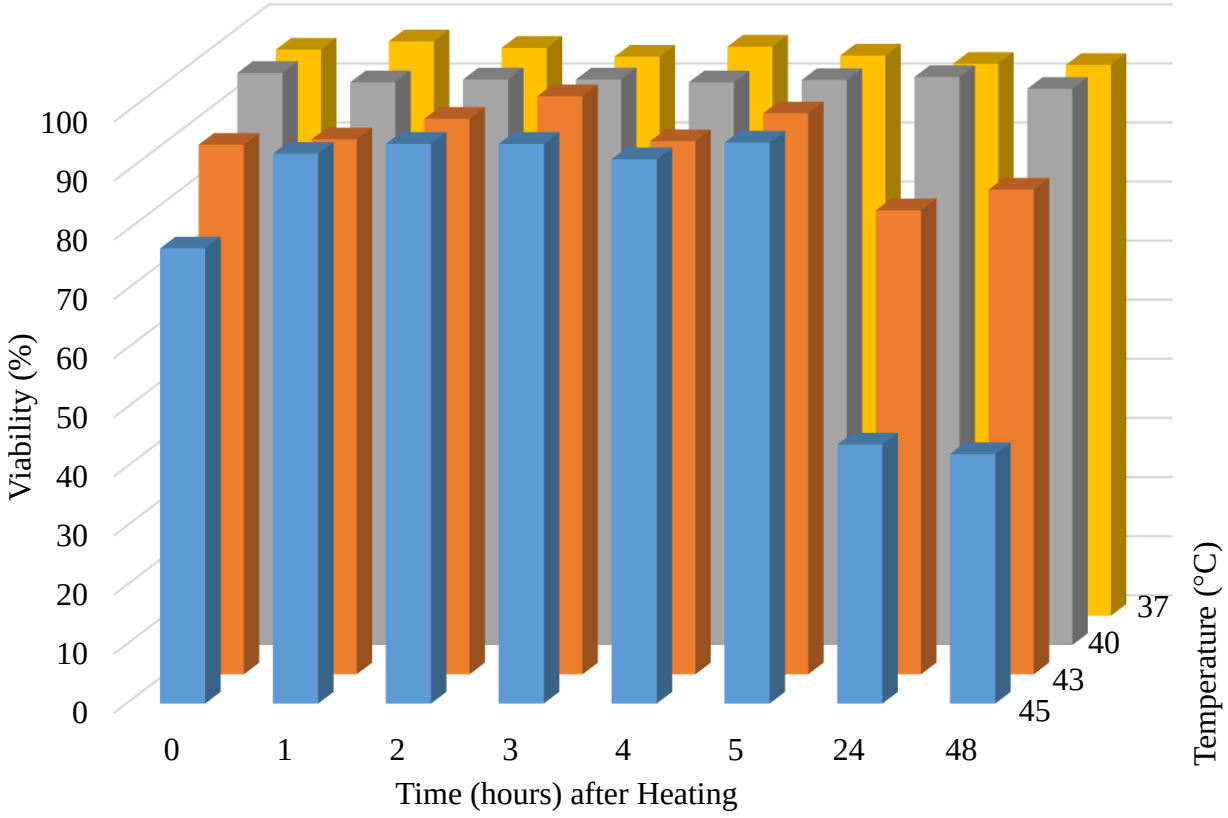


Figure 4.5: Viability of cells after heating to 45°, 43°, 40°, and 37° C and holding temperature for 2 minutes. Viability was determined at hours 0, 1, 2, 3, 4, 5, 24, and 48. N=3 samples per combination of time and temperature.

Initial experiments were designed to find combinations of parameters that would make sense to use for molecular delivery work. One preliminary step was to determine conditions that allowed maximum current flow from the power supply at each of the voltages. This was achieved by determining minimum resistance that could be added to the circuit to control current flow (see Figure 3.5 this is the block diagram of the entire system) and still allow the voltage to be maintained. This was empirically determined for 10, 12, 14, and 16 kV and resulted in maximum currents between 300 and 400 μA. After this was determined, the cells were treated using each voltage (and maximum current) for 5 minutes and viability was determined immediately after treatment. For all voltages, viability was approximately 20-30% which was unacceptably low. More preliminary works were done by increasing the resistance incrementally, treating cells for 5 minutes, and then determining viability. For each voltage, the resistance was increased until the post-treatment viability was greater than 85%. This viability was chosen to show that the cells were still being affected by the corona charge while still maintaining a high enough viability that the molecular delivery data would not be compromised. All of the voltages showed an 85% viability, or above when between 50-65μA of current was flowing in the system. Table 4.1 shows the combination of voltage, current, and current limiting resistance to achieve this.

Table 4.1: Combination of Voltage, Current, and Current Limiting Resistance that Resulted in 85% or Greater Cell Viability

Voltage (kV)	Current Measured (μA)	Current Limiting Resistance (MΩ)
10	63.83	42.5
12	64.67	70
14	56.00	120
16	51.33	170

4.1.4 Molecular Delivery of Sytox™ Using Corona Charge and Ambient Temperature

After corona charge generating parameters that resulted in over 85% viability were determined (section 4.1.3 above) for 10, 12, 14, and 16 kV. Sytox™ was delivered to Jurkat cells. Sytox™ was added to the cells immediately after they were treated with corona charge using the parameters in Table 4.1. The resulting fluorescence increases were quantitated using the fluorescent plate reader. The resulting data are shown in Table 4.2. Three samples were treated for each set of conditions shown in the table. Each corona charge treated sample had an associated control sample that received not charge but was exposed to Sytox™. The mean fluorescence (arbitrary units) data 10 minutes post-treatment was computed for like treated samples as well as control samples. In addition, the difference between each corona charge treated sample and its respective control sample was calculated. The mean of these differences (and standard deviation) is also shown in the table. Note that N is equal for the control and the treated data at the same voltage.

Table 4.2: Mean Fluorescence and Standard Deviation Data for Corona Charge Treatment

		Control		Treated		Difference	
Voltage (kV)	N	Mean Fluorescence	Standard Deviation	Mean Fluorescence	Standard Deviation	Mean Fluorescence	Standard Deviation
10	6	923.44	173.42	1353.00	131.14	429.56	92.55
12	7	770.95	273.59	1115.38	271.28	344.43	126.04
14	7	690.67	177.60	1028.05	196.58	337.38	123.02
16	3	1055.33	135.98	1566.33	77.55	511.00	67.21

Based on this data, it would be easy to conclude that 16 kV produced the highest amount of delivery based on mean fluorescence. However, note that there is a considerable difference in the mean values of the control samples that range from approximately 690 to 1055. Control samples were the highest for samples treated with 16 kV. This range of values can be attributed to variation in the cells from day to day as the data in Table 4.2 were collected over several days

using Jurkat cells that were grown on different days. Other factors could have played a role including the length of time that cells were stored in PBS. This can affect the permeability of the cells to Sytox™ as shown in Figure 4.2. This data reinforces the value and need to have a control sample for each treated sample. The analysis first focused on determining if each of the corona charge treatment parameters resulted in significantly different mean fluorescence values relative to their controls.

Table 4.3: T-Test Data for Comparing the Fluorescence of Corona Charge Treated Samples to Control Samples

Voltage (kV)	Degrees of Freedom	T_{stat}	$T_{critical}$
10	10	-4.84	2.23
12	12	-2.36	2.18
14	12	-3.37	2.18
16	4	-5.65	2.78

The absolute value of the T_{stat} for each set of corona charge generating parameters (voltage) was greater than the $T_{critical}$. Therefore, each of the corona generating parameters resulting in statistically significant cellular uptake relative to their respective control samples. Next, the fluorescent data from each set of parameters were compared to each other. Since the fluorescence of the control samples did vary from day to day and experiment to experiment the difference between the treated and control samples was used for analysis. Table 4.2 shows the data used, and Table 4.4 shows the resulting comparison. The absolute value of the calculated T_{stat} values was not greater than $T_{critical}$ for any of the comparisons in the table. Thus, there was no statistically significant difference between the molecular delivery of Sytox™ for a 5 minute treatment time at ambient temperature using any of the applied voltages. Since there was no apparent benefit to using any of the voltages investigated, the lowest voltage (10 kV) was used in subsequent experiments.

Table 4.4: T-Test Comparing the Fluorescence Differences Obtained Using Different Corona Charge Generating Parameters

Voltage (kV)	Degrees of Freedom	T_{stat}	$T_{critical}$
10 v 12	11	1.37	2.20
10 v 14	11	1.50	2.20
10 v 16	7	-1.34	2.36
12 v 14	12	0.11	2.18
12 v 16	8	-2.11	2.31
14 v 16	8	-2.25	2.31

4.1.5 Influence of Electrical Current on Corona Charge Mediated Delivery of Sytox™ at 10kV, Ambient Temperature, and a 5 Minute Treatment Time

Corona charge based delivery experimentation up to this point indicated that a 5 minute treatment time with corona charge generated using 10 kV would be suitable at ambient temperature. However, the influence of electrical current had not yet been investigated. So, experiments were conducted to determine if current caused a statistical difference in cellular uptake of Sytox™. Table 4.5 shows the combinations of voltage, current, and current limiting resistance tested.

Table 4.6 shows the means and standard deviations of the resulting data for treated samples and their respective control samples. The difference between the control and treated samples is also shown in the table. Table 4.7 shows the T-test data used to compare the controls to the treated. Each of the different currents resulted in statistically different Sytox™ delivery based upon the T-test data because the absolute value of all the calculated T_{stat} values was greater than their respective $T_{critical}$ values. Table 4.8 shows T-test data for the difference data. This showed that there was a statistical difference between the currents, with the exception of between 15μA and 25μA. 75 μA provide the highest mean fluorescence difference and was statistically different from the data from samples treated with all other currents. Thus, "optimal" corona

charge treatment conditions at this point in the experimental scheme appeared to be a 10 kV corona generation voltage, 5 minutes treatment time, and 75 µA or current at ambient temperature.

Table 4.5: Combination of Current, Voltage, and Current Limiting Resistance Used for Sytox™ Delivery

Current (µA)	Voltage Observed (kV)	Resistance (MΩ)
15	10.3	150
25	10.3	100
50	10.3	41
75	10.3	26

Table 4.6: Mean Fluorescence and Standard Deviations for Current Tested

		Control		Treated		Difference	
Current (µA)	N	Mean Fluorescence	Standard Deviation	Mean Fluorescence	Standard Deviation	Mean Fluorescence	Standard Deviation
15	5	656.87	78.19	857.07	77.17	200.20	38.46
25	6	662.72	92.10	898.06	88.71	235.33	85.97
50	3	692.22	39.21	1008.33	31.18	316.11	61.70
75	6	708.72	76.51	1245.06	111.66	536.33	68.83

Table 4.7: T-Test Data for Comparing the Fluorescence of Corona Charged Treated Samples to Control Samples Resulting from Varying Current

Current (µA)	Degrees of Freedom	T_{stat}	$T_{critical}$
15	8	-4.07	2.31
25	10	-4.51	2.23
50	4	-10.93	2.78
75	10	-9.71	2.23

4.1.6 Cell Viability as a Function of Corona Charge Treatment Time Using 75µA of Current at Ambient Temperature

75 µA of current produced the highest fluorescence that after compensating for the fluorescence of the control samples was statistically different from all of the other currents

investigated. Corona charge using this level of current and 10 kV at ambient temperature was used in combination with 1, 2, and 5 minute treatment times. The goal was to determine cell viability post-treatment. The experimental protocol was similar to when the effects of heat alone on viability were examined; however, cells were treated only with corona charge instead of heat. In one experiment, viability was tested every hour for five hours and then again at 24 hours. For another experiment, viability was assessed at time 0, 24 hours, and 48 hours. Experimental data was combined and is shown in Figure 4.6. Table 4.9 shows a summary of the cell viability curves. The figure shows that there is little change in viability for 5 hours. However, the figure and the table both indicate that there are dramatic and unacceptable reductions in viability at 24 and 48 hours (80 percent reduction or greater) for treatment times of 2 and 5 minutes. A 1 minute treatment time resulted in better results in that the 24 and 48 hours viabilities ranged from approximately 75-80%. One explanation could be that corona charge caused apoptosis; however, further experimentation would be required to confirm this. While 75 μA (along with 10 kV) showed the highest molecular delivery the low cell that results would likely negate any delivery effects as cells must remain alive to express delivered DNA and to divide. Efficient nucleic acid delivery is one goal of this work.

Table 4.8: T-Test Data for Comparing the Fluorescence Differences Between the Corona Charged Treated to Control Samples Resulting from Varying Current

Current (μA)	Degrees of Freedom	T_{stat}	$T_{critical}$
15 v 25	9	-0.84	2.26
15 v 50	6	-3.34	2.45
15 v 75	9	-9.68	2.26
25 v 50	7	-1.43	2.36
25 v 75	10	-6.69	2.23
50 v 75	7	-4.66	2.36

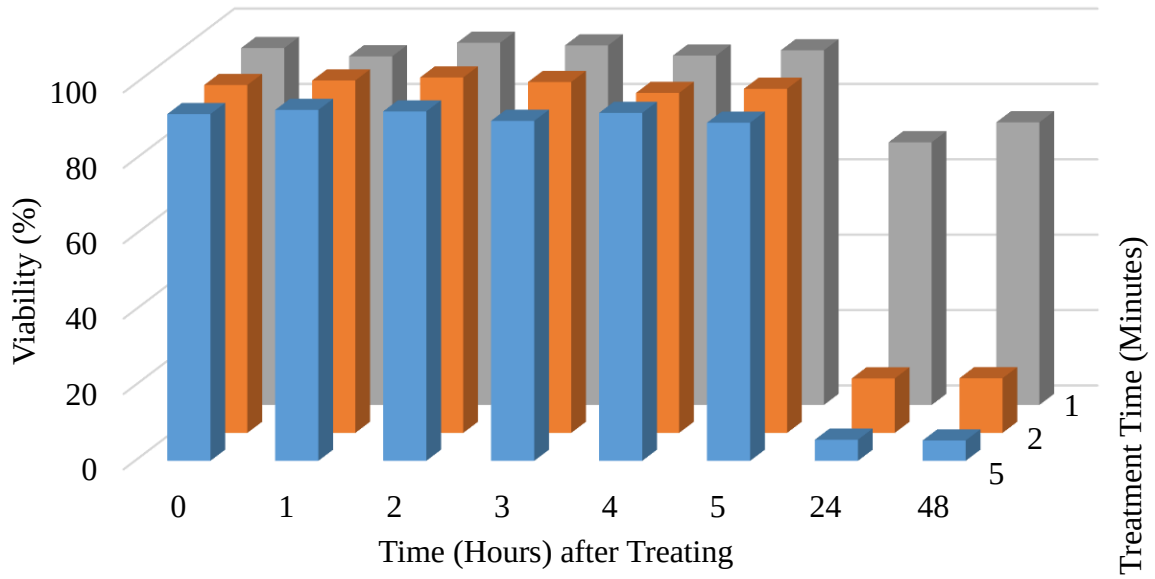


Figure 4.6: Viability of cells after treatment for 1, 2, and 5 minutes with corona charge generated using 10 kV with 75µA of current. N=3 samples per treatment time.

Table 4.9: Cell Viability after Being Treated Using 75µA

Time (Min)	0 Hours (%)	24 Hours (%)	48 Hours (%)
5	87.7	10.0	5.4
2	91.6	21.6	14.5
1	95.1	79.6	75.0

In an attempt to treat cells but maintain viability, the current was reduced to 25 µA by using a 100 MΩ current limiting resistor. Then, viability data was obtained for treatment times of 1, 2, 3, and 5 minutes (using 10 kV). The resulting data are shown in Figure 4.7. Numeric data for 0, 24, and 48 hours is shown in Table 4.10. Viability after treating for five minutes was very high for the first 5 hours; however, it reduced to below 40% after 24 and 48 hours. This showed that the treatment time was too harsh for the cells. In contrast, the figure and table indicate that

viability is well maintained after treating for 1, 2, and 3 minutes. Viabilities were about 95% at 24 and 48 hours for cells treated for 1 minute. The viability for 2 and 3 minute treatment times were about 80% at 24 and 48 hours. These were acceptable changes. Thus, treatment times of 1, 2, and 3 minutes were used to deliver Sytox™ to Jurkat Cells. Table 4.11 shows the resulting mean fluorescence data and standard deviations. It also shows the calculated difference between each sample and its control sample. Table 4.12 shows the T-test results between the control and the treated fluorescent units. The only treatment time that had a statistically different mean fluorescence when compared to its control was 3 minutes. This was the treatment time used for subsequent experiments. Consequently, the "optimal" corona charge parameters identified at this point in the study were 3 minutes of treatment time, 25 μA, and 10 kV at ambient temperature. These parameters were ultimately combined with moderate heating.

Table 4.10: Cell Viability for Corona Charge Treatment at 25μA

Time (min)	0 Hours (%)	24 Hours (%)	48 Hours (%)
5	96.0	38.8	27.9
3	94.6	79.4	78.1
2	94.8	80.3	82.2
1	94.0	94.3	95.5

Table 4.11: Mean Fluorescence and Standard Deviations of Samples Treated with 25μA

		Control		Treated		Difference	
Time (min)	N	Mean Fluorescence	Standard Deviation	Mean Fluorescence	Standard Deviation	Mean Fluorescence	Standard Deviation
3	3	750.67	39.40	837.22	7.24	86.56	32.22
2	3	725.78	69.75	822.33	113.54	96.56	49.10
1	3	584.22	17.26	556.22	69.10	-28.00	52.68

Table 4.12: T-Test to Compare Control to Treated Varying Time

Time (min)	Degrees of Freedom	T_{stat}	$T_{critical}$
3	4	-3.74	2.78
2	4	-1.26	2.78
1	4	0.68	2.78

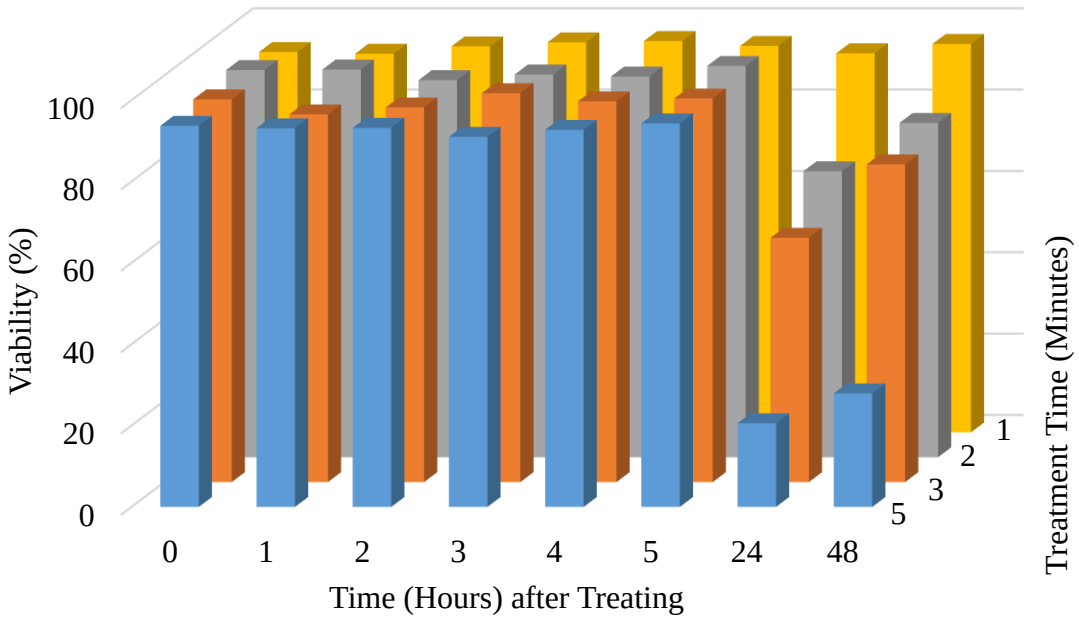


Figure 4.7: Viability of cells after treatment for 1, 2, 3, and 5 minutes with corona charge generated using 10 kV with 25μA of current. Viability was determined at hours 0, 1, 2, 3, 4, 5, 24, and 48. N=3 samples per treatment time.

4.2 Effects of Heating and Treating with Corona Charge Treatment

4.2.1 Effects of Heating for 3 Minutes on the Cellular Uptake of Sytox™

Before the "optimal" ambient temperature parameters, from section 4.1.2, could be combined with heat to determine if there are any synergistic effects, the potential effects of heat on Sytox™ uptake were investigated. Control samples were kept at room temperature while heated samples heated to either 37°, 40°, or 43° C and held there for 3 minutes. 45°C was not investigated further as it resulted in unacceptable viability reductions (section 4.1.2). The 3 minute hold time corresponded to the "optimal" corona charge treatment time (for ambient temperature) identified above. Thus, the two times corresponded. Table 4.13 shows the mean and standard deviations of the control and heated samples in arbitrary fluorescent units. Table 4.14

shows the T-test data used to compare the control to the heated cells. Numerically, the data in Table 4.14 show no or very small increases in mean fluorescence that resulted from heating samples to any of the 3 temperatures for 3 minutes. None of the combinations of temperature and hold time resulted in statistically significant differences between the treated and the control sample. Thus, it can be concluded that none of these three sets of temperature and time parameters will statically increase Sytox™ delivery when used alone.

Table 4.13: Mean Fluorescence and Standard Deviations for Control and Heated Samples

		Control		Heated	
Temperature (°C)	N	Mean	Standard Deviation	Mean	Standard Deviation
37	3	689.00	98.56	749.67	39.30
40	3	752.67	84.81	832.89	21.57
43	3	791.78	28.73	800.33	49.42

Table 4.14: T-Test Comparing Control to Heated Varying Temperature

Temperature (°C)	Degrees of Freedom	T_{stat}	$T_{critical}$
37	4	-0.99	2.78
40	4	-1.59	2.78
43	4	-0.26	2.78

4.2.2 Effects of Heating and Treating with Corona Charge for 3 Minutes on the Cellular Uptake of Sytox™

At this point in the study, it was determined that a corona charge treatment time of 3 minutes would be used with an applied voltage of 10 kV and a current of 25 µA. Before treating cells with corona charge they would first be heated to 37°, 40°, or 43°C and held at their respective temperatures for the 3 minute treatment time. Immediately after the corona charge treatment, Sytox™ was added to the cells and spectrofluorimetric analysis was started.

Alternatively, no Sytox™ was added and the cells were incubated and viability was assessed periodically. Table 4.15 shows the specifics of the treatment parameters.

Table 4.15: Final Parameters Chosen for Molecular Delivery and Viability Assessment

Voltage (kV)	Current (μA)	Resistance (MΩ)	Distance (cm)	Time (min)	Temperature (°C)
10	25	100	1	3	Room Temp
10	25	100	1	3	37
10	25	100	1	3	40
10	25	100	1	3	43

Viability of cells that were treated with plasma and heated to 37°C, 40°C, and 43°C was assessed at hour 0, 24, and 48 post-treatment. Based on viability evaluations earlier in this study, viability did not change much within the first five hours. Changes were typically noted at 24 and 48 hours. Figure 4.8 shows the results.

Viability generally remains relatively high after being heated and treated with corona charge. The largest decrease was noted for 43°C. This was expected because it showed the highest cell death with heating alone.

Mean fluorescence and standard deviation data are shown in Table 4.16 for control samples, treated samples, and the difference (treated minus control). Values used for statistical comparison of the fluorescence data from the samples to the data from the control samples are shown in Table 4.17. For 37° and 40°C, the treated samples (heat and corona charge) were statistically different than their respective control samples (heat only). The Fluorescence of samples treated at ambient temperature (no heat and plasma) compared to their controls (no treatment) also showed statistical significance. Data from 43°C was not statistically different. Given that heat alone did not result in significant increases in Sytox™ uptake it can be concluded that the plasma is causing a positive and statically relevant effect.

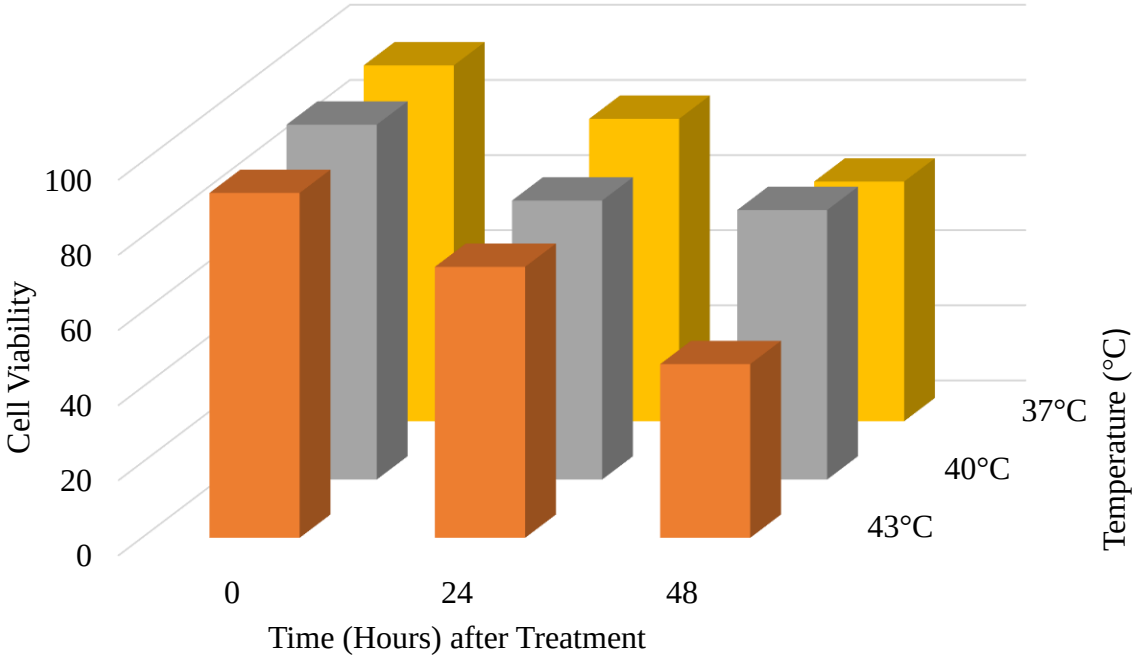


Figure 4.8: Viability of cells after 3 minutes of being treated using 25μA and heated to 37°, 40°, and 43°C. Viability was determined at hours 0, 24, and 48. N=3 samples per temperature.

Table 4.16: Mean Fluorescence and Standard Deviations for Control and Treated at Varying Temperatures

		Control		Treated		Difference	
Temp (°C)	N	Mean Fluorescence	Standard Deviation	Mean Fluorescence	Standard Deviation	Mean Fluorescence	Standard Deviation
RT	3	750.67	39.40	837.22	7.24	86.56	32.22
37	9	717.04	48.12	844.04	76.45	127.00	87.03
40	14	745.83	90.80	938.98	87.31	193.14	122.71
43	8	1040.25	155.90	1165.29	166.25	125.04	77.91

Table 4.17: T-Test Comparing Control to Treated Varying Temperature

Temperature (°C)	Degrees of Freedom	T_{stat}	$T_{critical}$
Room Temp	4	-3.74	2.45
37	16	-4.22	2.12
40	26	-5.73	2.03
43	14	-1.55	2.14

Since treating with corona charge (25μA, 10 kV, 3 minutes) combined with heating to 40°C resulted in statistically significant data, an additional experiment was performed at that temperature. It was very similar to the previous experiments, except that the control was kept at room temperature rather than being heated to 40°C. Mean fluorescence and standard deviation data are shown in Table 4.19 for control samples, treated samples, and the difference (treated minus control) for 40°C when the control was kept at room temperature. The room temperature data was also included to compare later on.

Table 4.18: Mean Fluorescence and Standard Deviations for Control and Treated at Varying Temperatures

		Control		Treated		Difference	
Temperature (°C)	N	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation
40	10	927.67	234.81	1275.50	282.28	347.83	185.57

Based on the data in Table 4.19, the control held at room temperature and the cells that were heated to 40°C and treated with corona charge at the optimal parameters were compared. The differences from the room temperature treated with corona charge and the 40°C were compared. The data shows that there is a statistical difference between the control at room temperature and then treated with corona at 40°C. There is also a statistical difference between the treated with corona and the treated at 40°C differences.

Chapter 5 Conclusions

The goal was to come up with a non-contact method to deliver molecules to a Jurkat cell (T-cell). This was done by determining whether corona, elevated temperature (heat), and a combination of the two had an effect on molecular delivery in Jurkat cells. This was accomplished by using a system that included a novel cell culture dish that could be futher developed into a commercial product. The cell culture dish allowed for high voltage to be constrained to the cell treatment area using matte enamel insulation, electrical tape, and Kapton. Eventually, it is envisoned that the dish could be produced as a single use sterile item, which would allow for the cells to grow and be seen under the microscope in the same dish. This can allow for less transfer of cells between dishes which will help maintain viability and facilitate the maitence of aseptic growth conditions.

While there may have been other combinations that would have produced a better delivery of Sytox™, a caveat was that cells had to survive after treatment so that they could be used for therapy. This led to the identification of 10kV, 25μA, and 3 minutes as parameters because they provided statistically significant differences in Sytox™ delivery as well as maintained cell viability of around or above 70% 48 hours after treatment. In addition to room temperature, the temperatures 43°, 40°, and 37°C were chosen because they maintained viability above 70% after 48 hours.

With these parameters, it was determined that corona charge alone had a statistically significant increase in molecular delivery at room temperature. Heat provided a higher molecular delivery from compared to the room temperature samples that were not treated with corona

charge, but it was not a statistically significant difference. Combined corona charge treatment and heating to 37° and 40° C resulted in a statistically significant increase in molecular delivery compared to controls that were only heated. Additionally, combined corona charge treatment and heating to 40°C when compared to a control at room temperature, showed a statistically significant increase in molecular delivery in comparison to a sample that underwent corona charge treatment at room temperature.

5.1 Future Work

Now that it has been determined that corona charge and the combination of heat and corona charge have a positive effect on molecular delivery in T-cells, there are more parameters that can be tested and more improvements that can be made to the process. The literature suggests that pulsing can have a positive effect on corona charge delivery in mammalian cells (Chelsea M. Edelblute, Heller, Malik, Bulysheva, & Heller, 2016; Sakai et al., 2006). There is also evidence to suggest that generating charge in a different environment such as helium or a mix of gases could have an effect as well (Richard J. Connolly et al., 2012; Richard J. Connolly et al., 2015; Richard J. Connolly et al., 2011; Jinno et al., 2017; Lee, Yi, Chung, & Yeom, 2001). Another change that could have a positive impact is the number of needles used. Currently, only a single needle was used, but there have been plasma generators made with multiple needles. This could potentially allow the corona charge to reach a wider area (more cells) in one treatment.

There are two main ways of optimizing the design of the dishes that are envisioned as imrovements in addition to making them sterile and disposable. Currently, the insulation used is a spray-on enamel from Home Depot, but an antimicrobial porcelain enamel coating could be used to help insulate the dish better (Michael Wilczynski, 1999). Or, the dish could be designed

without a need for an enamel coating by only coating the ell treatment area with gold. Furthermore, one way to make the dish more economically would be to use a thinner layer of gold. A 100 Å layer of gold was used with an underlayer of chrome, but that was to ensure that the dishes could be used for weeks at a time with constant treatment. The dishes that would eventually be used would just have a thin layer of gold. This is to ensure that the cells could be treated, but also so the dish could be used under a microscope and the cells could be seen.

One day, this system can hopefully be used in more and more hospitals. This would ensure that patients, especially children, would not have to travel far from their homes to receive lifesaving treatment. This would save on cost for the patient and also bring in more business to certain hospitals. This would also allow for the treatment to become more widespread and available. With more people using the treatment and the transportation of cells decreasing, it's possible the price of the therapy would also go down. Currently, one in eight children diagnosed with cancer do not survive. On top of this, 60% of the children who do survive who have undergone the current methods of treatment (chemotherapy, radiation, etc.) suffer late-effects, including being diagnosed with a different type of cancer ("Important Facts," 2018). CAR-T cell therapy can not only save lives but also improve the quality of life after cancer. With this procedure, it could become more widespread and possibly save more children from cancer.

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About the Author

Molly Skinner received her Bachelor's degree in Chemical Engineering at the University of South Florida in May of 2017. She began her research career at the University of Florida in 2011 when she was 16 by studying polyhydroxyfullerenes. While in attendance as an undergraduate at the University of South Florida, she conducted research in hydrogels and using natural polysaccharides to combat neurodegenerative diseases such as Alzheimer's. She presented her Alzheimer's research at the University of South Florida's Undergraduate Research Colloquium and was one of thirty to receive the Excellence in Undergraduate Research Award. She later went on to present the research as a National Finalist in the WE18 Poster Competition. During the summer of 2018, Skinner took part in an NSF fellowship called the International Research Experience for Students at the National University of Singapore. While there, she used hydrogen-deuterium exchange mass spectrometry to study how osmolytes affect the structure of viruses, specifically the Turnip Crinkle Virus. With this research, she received first place at the Tampa WELocal Collegiate Coemption.

Skinner continues to be a highly involved member in her university's sections of the Society of Women Engineers and American Institute of Chemical Engineers. As an undergraduate, Skinner held many officer positions in SWE, including Treasurer her senior year. Along with her involvement in academic societies, Skinner also founded a nonprofit. She is the Secretary for the SkinnerStrong Foundation, a foundation dedicated to raising money for childhood leukemia research. The foundation was created in honor of Skinner's late brother, who passed away while being treated for Hypodiploid Acute Lymphoblastic Leukemia.