NANOPARTICLE MEDIATED ABLATION OF BREAST CANCER CELLS USING A NANOSECOND PULSED ELECTRIC FIELD

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The undersigned, appointed by the Dean of the Graduate School, have examined the thesis entitled

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Presented by Christopher Burford

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And hereby certify that in their opinion it is worthy of acceptance.

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NANOPARTICLE MEDIATED ABLATION OF BREAST CANCER CELLS USING A NANOSECOND PULSED ELECTRIC FIELD

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ABSTRACT

In the past, both nanomaterials and various heating modalities have been researched as means for treating cancers. However, many of the current methodologies have the flaws of inconsistent tumor ablation and significant destruction of healthy cells. Based on research performed using constant radiofrequency electric fields and metallic nanoparticles (where cell necrosis is induced by the heating of these nanoparticles) we have developed a modality that simlarly uses functionalized metallic nanoparticles, specific for the T47D breast cancer cell line, and nanosecond pulsed electric fields as the hyperthermic inducer. Using both iron oxide and gold nanoparticles the results of our pilot studies indicated that up to 90% of the cancer cells were ablated given the optimal treatment parameters. These quantities of ablated cells were achieved using a cumulative exposure time 6 orders of magnitude less than most in vitro radiofrequency electric field studies.

1 INTRODUCTION:

1.1 Breast Cancer

Malignant cancers, even if diagnosed early, are still difficult to treat. One of the most prevalent and deadly forms of cancer is breast cancer. Breast cancer is most commonly diagnosed in women, but it can rarely be found in men as well.

Based on the most recent review, 12.4 percent of women will develop breast cancer during their lives [National Cancer Institute]. In other words, 1 out of every 8 women will receive a breast cancer diagnosis in their lifetime. In 2011 alone, there were over 230,000 new cases of invasive breast cancer, and over 57,000 new cases of non-invasive breast cancer. Although it is a much smaller number, there were also over 2,100 new cases of invasive breast cancer in men in 2011. In the United States, in women, breast cancer is the second most commonly diagnosed cancer besides skin cancer, and among cancers it holds the second highest death rate, falling only behind lung cancer [BreastCancer.org].

There are several risk factors that increase a woman's risk of developing breast cancer. The most dominant of these factors is age. At age 30, any given woman has a 0.44 percent chance of developing breast cancer, at age 40 the chance increases to 1.47 percent. This trend continues up to age 70 and beyond where a woman has a 3.82 percent chance to develop breast cancer.

These percentages by age are based on the entire female population. There are several additional risk factors that can drastically increase the likelihood of a woman developing breast cancer [National Cancer Institute].

One major risk factor is genetic mutations. Certain breast cancer genes (BRCA1 and BRCA2) can mutate and thus, greatly increase one's chances of developing cancer. Another factor is breast density. The milk-producing tissue of the breast is characteristically dense; whereas, the fatty tissue of the breast is much less dense. Women who possess higher ratios of dense to less dense tissue in their breasts are more likely to develop breast cancer. Another factor that plays tremendous risk is family history. A woman who shares the same genetic line with relatives who have developed the deadly disease is at an increased risk to also develop cancer. Similarly, women who have already been diagnosed with, and beaten breast cancer once are at a higher risk to develop a second tumor than a woman who has never been diagnosed [National Cancer Institute].

Other additional factors that have been studied that increases the risk for developing breast cancer are: exposure to radiation for other treatment, increased alcohol consumption, early age menstruation, long-term menopausal hormone therapy, obesity, low levels of physical activity, and race (white women are at a higher risk) [National Cancer Institute].

1.2 Current Thermal Treatments

One strategy used clinically and also extensively in research for treating breast cancer and other cancers is hyperthermia, or destruction via thermal energy. The most common mechanisms by which hyperthermia decimates cancer cells are protein denaturation or by causing irreparable damage to the cell membrane [Day, 2009]. Some common methods of generating the thermal energy required for hyperthermic cancer ablation include laser light [Chen, 1997], ultrasound [Jolesz, 2002], and microwaves [Seki, 1999]. The potential advantages of thermal treatments include minimal invasiveness, increased ease of operation, and the ability to reach cancerous legions that are unreachable via surgery. One obstacle to achieving hyperthermic ablation is that the source of the thermal energy may be required to penetrate through multiple layers of tissue before actually reaching the tumor. Moreover, the destructive energy alone is not selective for only the cancerous cells, an issue that often results in significant damage to healthy tissue [Day, 2009]. With the continued development in nanobiotechnology, nanoparticles have provided one option to improve both the selectivity and efficiency of thermal based cancer treatments.

1.3 Nanoparticle-Based Hyperthermic Treatments

1.3.1 Near-Infrared Photothermal Therapy (NIR)

One such application of nanoparticles is near-infrared (NIR) photothermal therapy. Standard photothermal interactions occur naturally when absorbers such as water, hemoglobin, oxyhemoglobin and melanin absorb light and convert it to heat energy. However, none of these absorbers provide any discrimination between healthy and non-healthy tissues. By incorporating a specific wavelength range, more specifically the near-infrared range from 700 nm to 900 nm, absorption by hemoglobin and water can be avoided [Van Gemert, 1995]. Furthermore, with the addition of an external absorber, more specifically an optically absorbing nanomaterial, these photothermal interactions can be used to specifically target cancerous tissues.

One type of nanomaterial used for near-infrared treatment are gold nanoshells. The structure of these nanoshells is a dielectric core with a very thin metal shell coating [Averitt, 1999]. Intravenously injected nanoshells accumulate at tumor sites without any selective biological molecules attached [James, 2007], but nanoshells are easily altered to include the additional selectivity provided by molecules such as antibodies [Lowery, 2006]. In a mouse model, NIR/nanoshell therapy has resulted in complete tumor regression after 3 minutes of 4 W/cm2 irradiation [O'Neal, 2004]. Furthermore, with NIR therapy, there is no significant heating of the surrounding tissues that are not treated

with nanoshells [Hirsch, 2003]. NIR treatements are also possible using other antibody-modified nanomaterials.

1.3.2 Magnetic Fluid Hyperthermia (MFH)

A second clinical application using nanoparticles for hyperthermia is magnetic fluid hyperthermia (MFH). In this treatment, magnetic nanoparticles, such as iron oxide, are either injected intravenously or directly into the tumor site. Once injected, an alternating magnetic field is applied to the tumor site. This process generates significant heat amongst the nanoparticles due to Néel and Brownian relaxations from the rotation of the magnetic moment inside the nanoparticle or from the same moment rotation of the nanoparticle as a whole [Wang, 2005]. Because of the alternation of the magnetic field, the magnetic moment of each particle changes so that it is in alignment with the magnetic field, and as the moment returns to equilibrium, the energy dissipated results in hyperthermia.

It has been demonstrated that MFH is effective in hyperthermically treating tumors [Hilger, 2005], and also that functionalizing the magnetic nanoparticles with a selective agent such as an antibody, results in larger temperature increases at the tumor site [Hilger, 2006]. One clinical study utilizing MFH involved the injection of iron-oxide nanoparticles into a prostate cancer patient. The study showed that after 6 weeks of treatment, the nanoparticles remained only in the tumor site for the entire duration of treatment, and that after exposure to an alternating magnetic field (100kHz and 4-5 kA/m)

temperatures were achieved that destroyed the cancerous cells. [Johannsen, 2005]. Clinical research suggests that MFH does not generate any effects detrimental to a patient's health, but the overall efficiency of the treatments need to be tested further.

1.3.3 Radiofrequency Ablation (RFA)

Another technique that has been expanded upon by the addition of nanoparticles is radiofrequency ablation (RFA). Standard RFA involves the insertion of a metallic probe directly into the tumor site, followed by the emission of a radiofrequency field, that generates destructive heat [Curley, 2001]. While standard RFA probing is effective in eliminating cancerous cells, it is not selective for only the cancerous cells and thus can cause significant damage to the healthy surrounding tissue. Using metallic nanoparticles instead of a metallic probe as the hyperthermic agent is one method currently being researched to improve the selectivity of radiofrequency treatment.

Three types of nanomaterials used in radiofrequency research include gold (AuNP), single walled carbon nanotubes (SWNT), and other magnetic materials (such as iron oxide). Gold nanoparticles have been shown to increase the temperature within a tumor after exposure to a radiofrequency field, and furthermore the amount of heat generated is directly related to the power of the radiofrequency field [Cardinal, 2008]. One study performed by Gannon et al. (2008) shows the profile for the heat generated by AuNPs at varying concentrations. Higher concentrations of nanoparticles generate higher

temperature increases. Additonally in this study, gold nanoparticles were non-selectively incubated with gastrointestinal cancer cells (Panc 1, Hep 3B) and exposed to a 13.65 MHz radiofrequency field for increasing time increments up to 5 minutes. In all samples treated with AuNPs, 96% of cells were ablated [Gannon, 2008]. A second study performed by Kruse et al. (2011) furthered the work done by Gannon to show that radiofrequency exposure times under 1 minute, the gold nanoparticles are much less effective at killing the cancerous cells. Results indicated that after 30 seconds of treatment, only approximately 20% of the cells were ablated; whereas, after 60 seconds, approximately 80% were found to be necrotic. Both of the previous studies utilized gold nanoparticles that were not functionalized with any sort of selective agent or antibody, thus limiting any potential *in vivo* significance. A third AuNP-radiofrequency study performed by Glazer et al. (2010) used functionalized gold nanoparticles with epidermal growth factor receptor (EGFR-1) conjugated to the surface. EGFR-1 is a trait overexpressed in the cell lines used (Panc-1, and Cama-1), and should provide for better attachment of the AuNPs to the cells. The results of this study, however, showed that the cell viability of Cama-1 varied minimally from before and after treatment, and Panc-1 retained almost 50% cell viability post treatment with radiofrequency [Glazer, 2010]. Comparing the results of the studies performed by Gannon et al. (2008) and Kruse et al. (2011) with the study performed by Glazer et al. (2010) would suggest free floating nanoparticles in the cell suspension (no direct contact with the cell membrane) are heated significantly enough

to cause some damage to the cells.

Another nanomaterial studied for radiofrequency nanoparticle ablation are single walled carbon nanotubes (SWNT). SWNTs exposed to a radiofrequency field generate heat through their resistive conductivity and electrochemical properties [Gannon, 2007]. Gannon et al. (2007) experimented *in vivo* with SWNT radiofrequency ablation using a rabbit model. Rabbits were injected with SWNTs directly into the tumor site and following radiofrequency treatment, evaluated histologically. Results indicated that there was significant cellular damage to the cancerous cells [Gannon, 2007]. Based on the two studies performed by Gannon et al. (2007, 2008) AuNPs seem to be more efficient at conducting radiofrequency energy into heat. Exposed to an equal power radiofrequency field of 600 W, AuNPs of concentration 11.19 mg/L experienced a temperature increase of 80°C; whereas, a 22 fold increase in concentration of SWNTs exposed for more than double the time only generated a 33°C temperature increase [Gannon, 2008; Gannon, 2007].

Magnetic nanoparticles have also been used as hyperthermic agents in radiofrequency studies. Xu et al. (2010) attempted to determine which magnetic nanomaterial was most effective in destroying cancer cells after exposure to a radiofrequency field. The materials tested included iron, iron/cobalt, and cobalt. After non-selective incubation (no antibody selectivity) with the cancer cells, samples were treated at 350kHz and 5kW from 2 to 45 minutes. After 2 minutes of exposure time, the amount of cell death recorded increased minimally. The results of the experiment indicated that iron nanoparticles

were most effective in ablating the cancer cells, reaching a necrosis level of approximately 70%. A subsequent radiofrequency study performed by Karmaker et al. (2011) utilized iron nanoparticles functionalized with epidermal growth factor (EGF). At a concentration of 5 μg/mL, only approximately 60% cells were deemed necrotic; whereas, at a much higher concentration of 20 μg/mL, over 90% of the cancerous cells were necrotic.

One apparent advantage of radiofrequency ablation is that its penetration depth into tissues is significantly deeper than near-infrared light. From 220 MHz to 85 MHz, radiofrequency energy penetration depth increases from 7 cm to 17 cm [Roschmann, 1987]; whereas, near-infrared light only reaches 1 cm into biological tissues, making it a much less appealing treatment option than radiofrequency.

1.4 Electromagnetic Field and Ultrasound Cancer Treatments

1.4.1 Microwave Hyperthermia

There are methods of treating cancers via hyperthermia that do not utilize nanoparticles. One such treatment option is treatment via microwaves. Microwave interaction with tissues generates thermal energy via the same mechanism as radiofrequency ablation, but at a significantly different frequency. Microwave treatments range from 0.3 GHz to 300 GHz. Some tissues, such as lung and bone, have low thermal conductivities. It is for tissues like

these, that microwave hyperthermia may be a more appealing option than radiofrequency ablation, because the frequency difference in microwaves is still capable of generating significant heat despite the low conductivity [Brace, 2009]. There is clinical data to suggest that microwave ablation is more effective in treating tumors larger in diameter than 5cm than no-nanoparticle mediated radiofrequency ablation, but microwave treatment is significantly limited in its ability to effectively treat tumors smaller than 5cm [Shibata, 2000]. Microwave hyperthermia still lacks significant clinical trials to generate a consensus on the effectiveness of the treatment.

1.4.2 Focused Ultrasound

Focused ultrasounds are mechanical waves with a frequency less than 20 MHz, and provide a promising option for treating cancers without nanoparticles in that they can generate destructive heat at any tissue depth in a non-invasive manner. Hyperthermia from ultrasound is generated from the absorption of acoustic energy. When focused, this energy is very high intensity and can be focused into a very small volume. One issue encountered with focused ultrasound treatments is that bubbles can form from the intense heat, and greatly lower the quantity of effective energy delivered to the tumor [Zderic, 2008]. As far as applying focused ultrasound, there are two modalities: constant application, and pulsed application. Results show that the temperature generated by constant application is up to 30°C greater than that of the pulsed application [Dewhirst, 1994]. Both can be used to

generate necrotic heat. Focused ultrasound is still more commonly used for other drug related medical practices, and its use in cancer ablation is still being developed.

1.4.3 Electrochemical Treatments

Electrochemical treatment (EChT) of cancers is a somewhat alternative approach to no-nanoparticle thermal treatment. In EChT, electrodes are inserted near the tumor site and a continuous low current is applied, with the resulting necrosis being achieved via electrolysis (electrically stimulated chemical reaction). This electrolysis creates vast pH changes that result in localized tumor necrosis. [Nilsson, 2000]. Studies such as the one performed by Turler et al. (2000) show that EChT is effective in causing some tumor regression, and that other cancer treatments such as chemotherapy can increase the effectiveness of EChT [Miklavcic, 1997]. Likewise with many other hyperthermic treatments, EChT is not selective for only the cancerous cells, making the positioning of the electrodes very critical, so that the maximal ratio of cancerous tissue/healthy tissue ablated is achieved. Although EChT is an affordable treatment, it is not widely used, and also it cannot be used for tumors located near major veins and arteries because of its non-selectivity [Metcalfe, 2007].

1.5 Electroporation Based Techniques

Electroporation techniques involve the exposure of cells to very quick electric field pulses that in turn cause the permeabilization of the cell membrane. The main use for electroporation is the delivery of an external drug or genomic sequence into the cell. There are also ways that electroporation can be used to treat cancers including electrochemotherapy (ECT), irreversible electroporation (IRE), and nanosecond pulsed electric fields (nsPEF).

1.5.1 Electrochemotherapy

Electrochemotherapy is a hybrid cancer treatment that combines the use of applied electricity with cancer fighting drugs. The drugs (most commonly bleomycin and cisplatin) used often have minimal visible effects on cells before uptake, but they have extreme toxicity after uptake by the porated cells. Cells become porated to facilitate the drug delivery by electrical pulses. The selectivity of the drugs combined with the fact that ECT targets only dividing cells (therefore cancer cells), means there is minimal damage to healthy surrounding tissues.

For ECT to be truly effective, all cancer cells in a tumor need to be permeabilized. Therefore, the parameters of the system need to be chosen so that the amplitude is high enough and the power low enough to ensure that the whole tumor is permeabilized, yet the procedure is also as safe as possible [Pavselj, 2005]. Electrodes comprised of either stainless steel or aluminum,

of three different designs are used for ECT. Plate electrodes treat shallow and surface tumors, needle electrodes are used for smaller tumors in difficult to reach regions, and needle hexagonal electrodes are used to treat larger tumors. [Mir, 2006]. The gold standard for electrical pulse application of ECT is based on the ESOPE study and is comprised of 8 pulses (square-wave) of 100μs duration, at a voltage of 1000V, and a frequency of 1 Hz or 5kHz [Marty, 2006].

Permeabilization of the cell membrane that facilitates ECT occurs via the following mechanism. The plasma membrane of all cells contains a resting electric potential. After the application of a pulsed electric field, eventually the transmembrane potential will reach a threshold that then causes the structural integrity of the membrane to be disrupted. This disruption forms pores that allow foreign molecules to enter into the intracellular region of the disrupted cells [Neumann, 1982].

As far as the drugs used for ECT, there are two most commonly used, bleomycin and cisplatin. Pron et al. (1993) demonstrated that bleomycin can enter cells without the aid of electric field pulses via a membrane protein, however, the cellular uptake via this mechanism is limited [Pron, 1999]. The addition of electric pulses to bleomycin treatment alters the cell membrane significantly, thus easily facilitating the uptake of the drug into the cells. A very small intracellular quantity of bleomycin is required to cause cell necrosis, only several hundred molecules [Poddevin, 1991]. Once internalized, bleomycin creates DNA that targets rapidly dividing cells (i.e. tumor cells),

but has little to no effect on non-dividing cells. This provides some level of selectivity to bleomycin/ECT treatment, a large advantage over competing treatments.

A second drug also used in ECT treatments is cisplatin. Unlike bleomycin, cicplatin is relatively effective in treating cancers without the addition of electric pulses [Gehl, 1998]. Cisplatin only demonstrates a 10 fold increase in effectiveness when electric pulses are applied; whereas, bleomycin's effectiveness is increased 1000 fold after electric pulse application [Orlowski, 1988]. The mechanism for cisplatin's effectiveness is similar to that of bleomycin in that the electric field pulses increase the ability of the cancer cells to uptake the drug, thus increasing the rate of necrosis in the tumor.

After *in vivo* electric pulse treatments, tissues undergo a change in blood flow that lasts for a few minutes. This reflex response of the nervous system to the electric pulses creates a "vascular lock" in the localized area of treatment [Gehl, 2002]. This "lock" can greatly benefit treatments such as ECT in that it will aid in keeping the drugs in the targeted area longer. Moreover, a study performed by Sersa et al. (1999) showed that this vascular lock can last multiple hours in tumor tissues, as compared to only minutes in healthy ones.

1.5.2 Irreversible Electroporation

Irreversible electroporation, or IRE, is another electric pulse based modality for treating cancers. In order to porate a cell, microsecond or millisecond duration electric pulses are applied to the cell, and the transmembrane potential generated creates small holes in the cell membrane that are often sufficient for intracellular manipulation. However, by altering the pulse properties, permanent poration that will eventually cause death is achievable [Davalos, 2005].

IRE has been utilized outside of the field of medical treatment for some time for the purpose of sterilizing liquids and other food related products [Beveridge, 2004]. However, the mechanism by which it kills cancerous tissues has only been researched more recently. Experimental variables that have an effect on the amount of the tumor destroyed include pulse duration, amplitude, and the number and frequency of the pulses. IRE has also been found to generate a vascular lock in the treated region [Gehl, 2002] similar to that achieved during ECT. IRE does not use thermal energy to cause cancer necrosis.

IRE also has a significant effect on tumor tissues, which physiologically have very different properties than normal tissues. In a mice model IRE study, Al-Sakere et al. (2007) achieved regression in 92% of tumors. This study demonstrated that cell necrosis was caused solely by extreme permeabilization of the plasma membrane of the cancer cells.

1.5.3 Nanosecond Pulsed Electric Fields

A third electroporation related means of inducing cancer necrosis is via nanosecond pulsed electric fields (nsPEF). Similar to IRE, the use of nsPEF is particularly appealing for treating cancers in that it does not require drugs, local heating, or chemical changes. Although voltages of several hundred kV/cm may be used during nsPEF, their durations are so short in time that any heating generated is extremely weak [Schoenbach, 2001]. Electric field pulses on the nanosecond time scale can influence both the plasma membrane of cells, and the intracellular organelles.

The means by which nsPEF can affect the intracellular organelles of cells is based on the required time to charge the cell membrane. After the application of a voltage to a cell, the cell membrane begins to charge, and eventually, an electric gradient forms across the cell membrane. The standard charging time of a mammalian cell is roughly 100ns [Tekle, 2005]. Any electric field pulse shorter than 100ns in duration will therefore cause no charge to accumulate at the cell membrane, and will solely influence the intracellular organelles. It has also been determined that pulses of duration 300ns or longer will exclusively influence and charge the plasma membrane and have no effect on the intracellular organelles. In other words, when researching nsPEF treatments, pulse duration has a significant impact on the mechanism behind any apparent results.

A study performed by Esser et al. (2009) proposed that exposure to nsPEF should create nanometer scale pores in the cellular membrane that

only small ions and molecules are able to enter through. Under these circumstances, the strength of the electric field inside the cell is the same as the applied electric field, and additionally, because of the size of the pores generated, the overall membrane permeability is affected little. It is difficult to design a nsPEF device that emits a truly consistent waveform considering the high voltages used in such short time durations.

Exposure to nsPEF, *in vitro* has resulted in various responses in many different types of cells, both cancerous and non-cancerous. It has been demonstrated in a study by Stacey et al. (2003) that cells in suspension are more susceptible to nsPEF pulses than those adhered to a surface. Also, studies executed by Ibey et al. (2010) and Hall et al. (2005) confirm that nsPEF pulses have only a small effect on the structural integrity of the cell membrane. However, intracellular organelles and their membranes can easily be porated by nsPEF exposure [Pakhomov, 2007]. Even a single electric pulse of nanosecond duration can generate significant effects such as calcium channel alteration or nanopore formation [Vernier, 2009; Nesin, 2011]. Pores formed from nsPEF exposure are not identical those formed from traditional electroporation [Nuccitelli, 2009]. Substances like Trypan blue, that easily cross the plasma membrane from traditional electroporation, are blocked from entering the cell after nsPEF exposure alone.

Although nsPEF treatments have been investigated *in vivo* in mice models, there are still many questions regarding the procedure. These include: the exact mechanism by which cell death is achieved, improving selectivity

between healthy cells and cancerous cells, and the general safety and efficiency of the treatments [Breton, 2012].

1.6 Our Approach

Based on the success of radiofrequency thermal ablation *in vitro* using metallic nanoparticles, and the potential for very short, yet effective treatment time using nanosecond pulsed electric fields, we have begun to research the combination of selective metallic nanoparticles with nsPEF for cancer ablation. Prior to experimentation, it was postulated that by attaching selective metallic nanoparticles to cancer cells, and then exposing them to nsPEF energy, significant heat would be generated amongst the nanoparticles so that the cancer cells would be damaged beyond repair. It was also believed, that if this heating modality was successful, it could achieve successful and comparable cell death as *in vitro* radiofrequency/nanoparticle studies in significantly less cumulative exposure time.

2 MATERIALS AND METHODS

2.1 Nanoparticle Preparation/Antibody Functionalization

Prior to any work being performed on the actual cancer cells, the nanoparticles had to be prepared/functionalized with antibodies. Two types of nanoparticles were used in these experiments: iron oxide and gold nanoparticles.

For the iron oxide nanoparticles, first, 100 μL of the stock solution of 0.1% w/v of 300 nm Fluorescent Carboxyl Magnetic Particles (Spherotech Inc., Lake Forest, IL) were incubated with 200 μL of 50 μg/mL of EDC and 50 μg/mL of NHS for 15 minutes. After the 15 minutes were concluded, the resulting solution was centrifuged for 5 minutes at 8000 rpm. After centrifugation, the supernatant fluid was extracted, resulting in a pellet of iron oxide nanoparticles. To this pellet of nanoparticles, 200 μL of DI water was added along with 3 μL of anti-EpCam antibody stock (Pierce Antibodies, Rockford, IL). This solution was then allowed to incubate and react for 2 hours. After the incubation period, the solution was centrifuged for 5 minutes at 8000 rpm. The supernatant fluid was again removed and the anti-EpCAM conjugated iron oxide nanoparticles were resuspended in 1 ml of 1x PBS.

For the gold nanoparticles, first, 50 μL of the stock solution of 54 nm Accurate Carboxyl Polymer Conjugated Spherical Gold Nanoparticles (NANOPARTZ Inc., Loveland, CO) were combined with 350 μL of 1x PBS in a 2 mL tube. Next, in a different 2 mL tube, 600 μL of 1x PBS was combined with 0.5

μL of anti-EpCam antibody stock (Pierce Antibodies, Rockford, IL). Both of these solutions were mixed separately, and then the gold nanoparticle/PBS solution was added to the tube containing the anti-EpCAM/PBS solution. This final solution was allowed to incubate while shaking for 1 hour.

2.2 Cell Preparation

The specific strain of cancer cells used for these experiments is the T47D breast cancer cell line (ATTC Number: HTB-133, University of Missouri Cell and Immunobiology Core, Columbia, MO). For each flask of cells used, the growth media was removed, and 5 mL of TrypLE Express (GIBCO, Life Technologies Corp.) was added in order to separate the cells from the bottom surface of the flask. The cells were then incubated in the TrypLE Express for 10 minutes. Now separated from the bottom of the flask, the cell solution was added to a 15 mL centrifuge tube and centrifuged at 1200 rpm for 10 minutes. The supernatant liquid was then removed and the cell pellet was resuspended in 500 μL of PBS and 500 μL of Accumax (Innovative Cell Technologies Inc., San Diego, CA). Next, 100 μL of the prepared nanoparticle solution (either iron oxide or gold) was added to the cell suspension and allowed to incubate for 1 hour. Following this incubation period, the solution now containing cell/nanoparticle conjugates was transferred to a 2 mL centrifuge tube and then centrifuged at 800 rpm for 10 minutes. After completion of the centrifugation, the cell pellet was resuspended in 1000 μL

of 1x PBS, and then centrifuged again at 800 rpm for 10 minutes. After this final centrifugation, the resultant pellet was suspended in 500 μL of 1x PBS and 500 μL of Accumax.

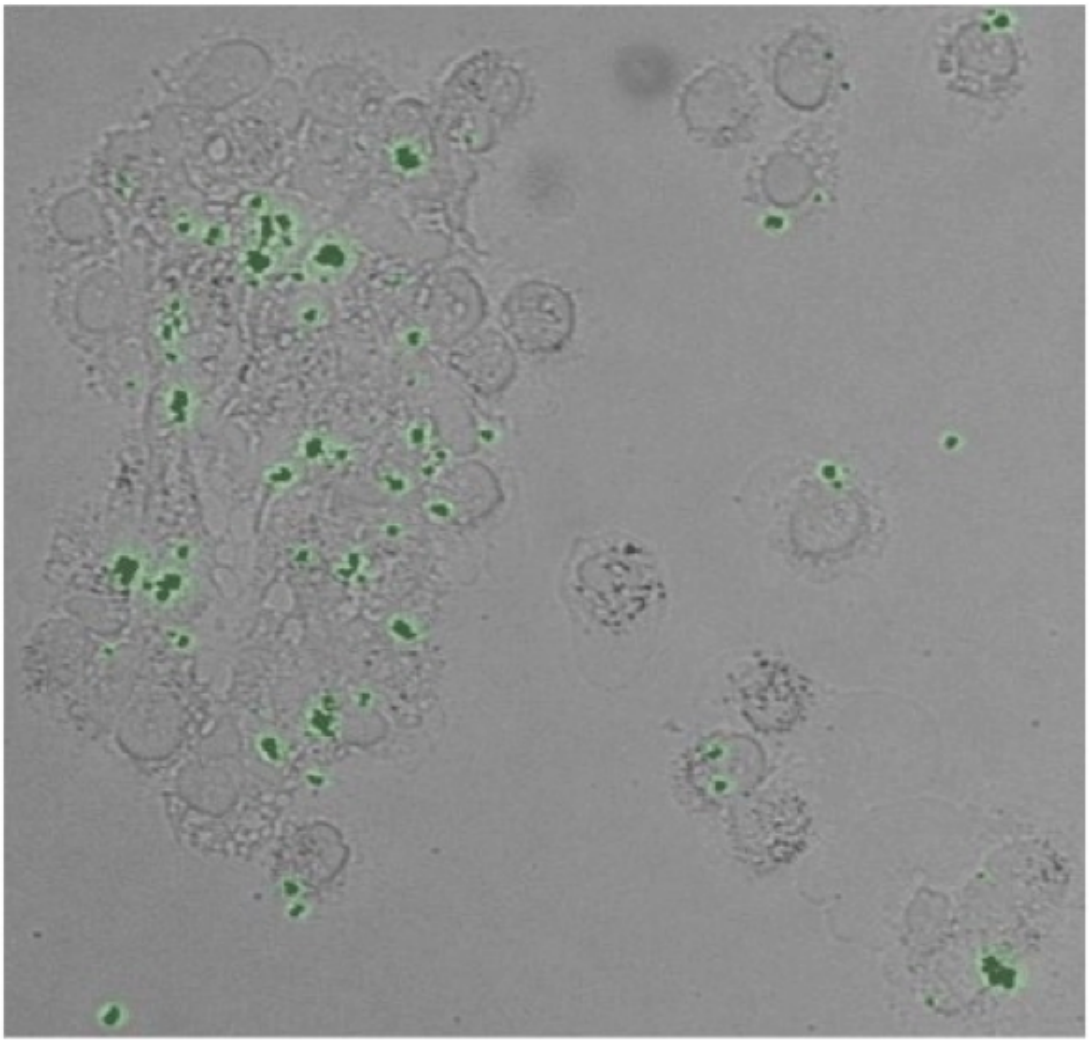

Figure 1: Relatively clean sample with almost every cell having at least one nanoparticle (fluorescent green on image) attached. There are very few free-floating nanoparticles in solution.

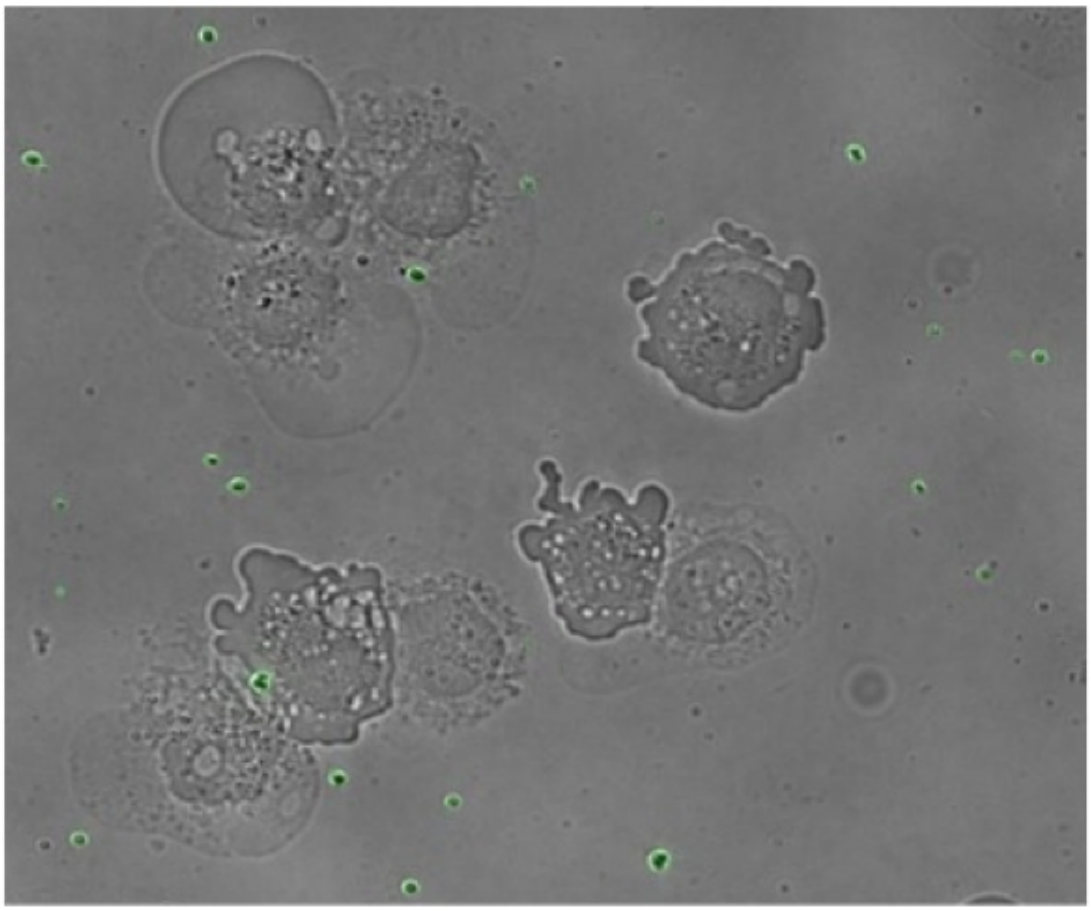

Figure 2: The cell preparation/nanoparticle conjugation procedure is not yet optimized resulting in some free-floating nanoparticles in suspension in some samples.

2.3 nsPEF Device and Treatment

Following the antibody functionalization of the nanoparticles, and the preparation of the cells, 100 μL of the solution containing cells with attached nanoparticles was then placed in each 0.1 cm electrode gap Gene Pulser Cuvette (BIO-RAD Laboratories Inc.). One at a time, each cuvette was then placed in the cuvette chamber of the nsPEF device and exposed to an arbi-

trary number of pulses within the following parameter ranges: 1-20 kV/mm electric potential, per pulse, and 60 ns or 300 ns pulse duration. Previous experimentation performed with the device demonstrated that the rise and fall time of each individual pulse is in the picosecond range. This duration of time is virtually instantaneous and causes no biological consequence during the transient change.

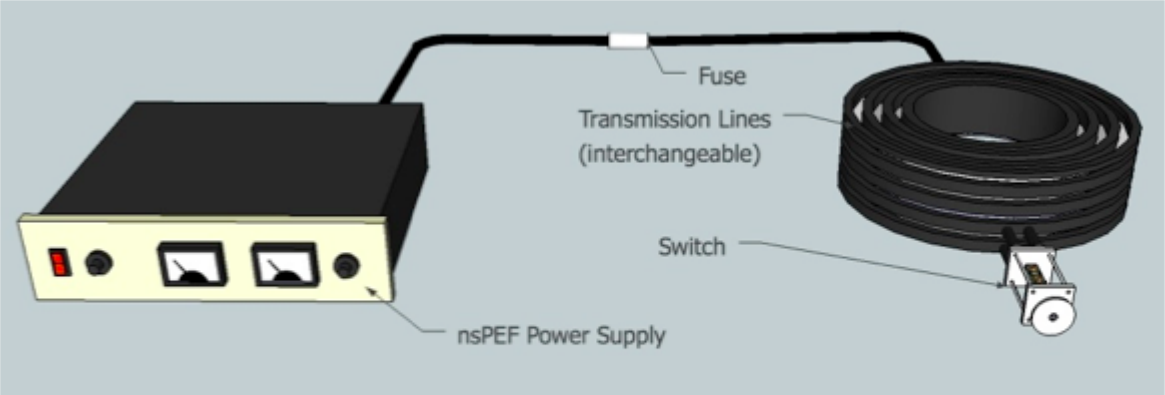

Figure 3: Schematic showing the nanosecond pulsed electric field device components: the power supply, having voltage options from 1 – 24 kV/mm (safe range) connects to interchangeable transmission lines (either 60ns or 300ns), which connect to the switch where the sample is located.

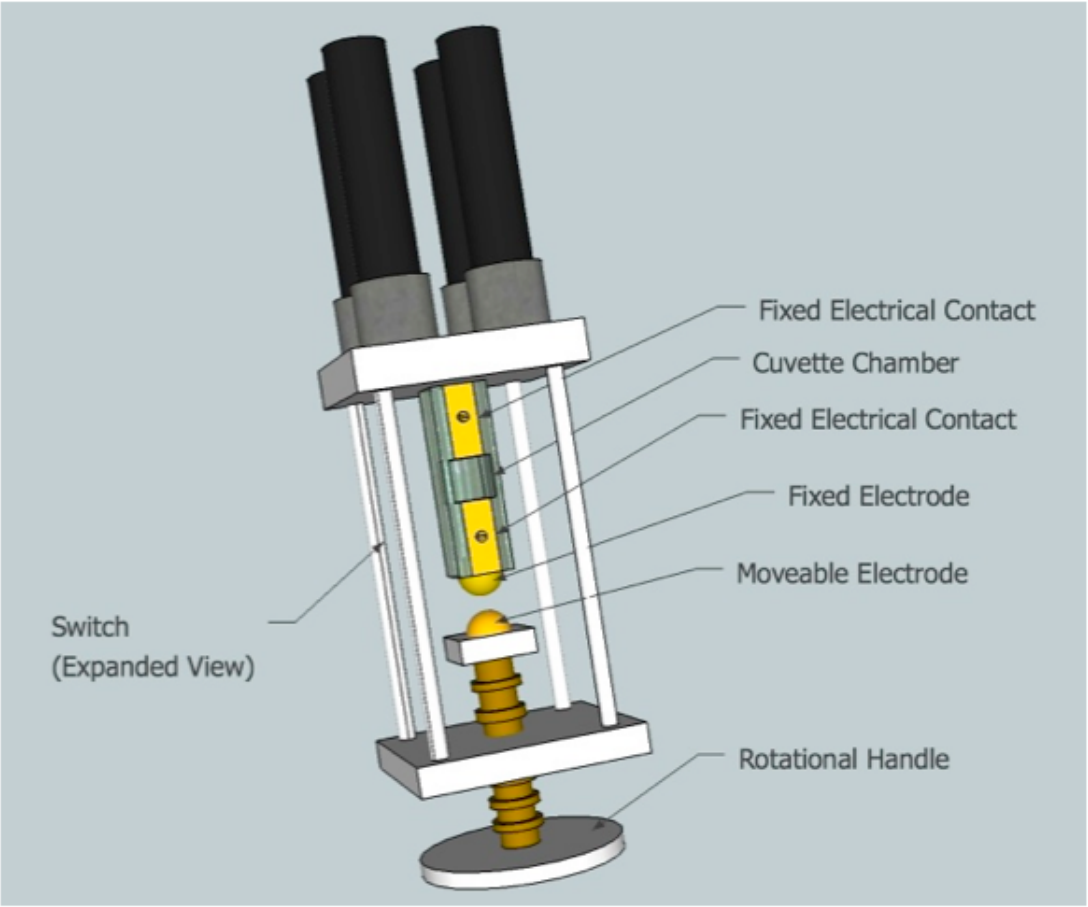

Figure 4: A close-up schematic of the switch component that shows the exact location of the sample (in the cuvette chamber), and the movable electrode that generates the nanosecond pulses when in close enough proximity to the fixed electrical components.

2.4 Cell Viability Evaluation

After treatment with the nanosecond electric field pulses, the viability of the cells must be analyzed in order to see if successful cell necrosis was achieved, and also to confirm that the control samples possessed high levels of cell viability. Each sample that was exposed to the nsPEF treatment was allowed to sit for 45 minutes. Afterward, 10 μL of Trypan Blue Solution and

10 μL of each sample were placed into corresponding 2 mL tubes. Then, 10 μL of each solution containing treated cells and Trypan Blue Solution was placed in a Bright-Line Hemacytometer (Hausser Scientific, Horsham, PA) and evaluated via light microscope. Based on the established protocol and equations for hemacytometer measurements, the cell viability of each sample was obtained.

3 RESULTS

3.1 Study Evaluating the Effect of 60ns and 300ns Pulsed Electric Field on T47D Cells without Metallic Nanoparticle Conjugation (Cells Only)

Before the effect of the nanosecond pulsed electric field on cells with metallic nanoparticles attached could be evaluated, the effect of the nanopulses on the cells alone needed to be determined. The viability of T47D cells without nanoparticle attachment was evaluated directly from the cell culture (no nanopulse exposure), and then compared with the viability of T47D cells that were treated with 8 nanopulses at 20 kV/mm of 60 ns. The viability of the sample taken directly from the cell culture was 96.6%, and the viability of the sample exposed to 8 nanopulses at 20 kV/mm of 60ns duration was 94.9%.

The viability of T47D cells without nanoparticle attachment from a different sample taken directly from the cell culture was then evaluated and compared with the viability of T47D cells treated with 8 nanopulses at 20kV/mm of 300 ns duration. The results of that experiment showed that the cell viability of the sample taken directly from the culture was 86.0%, and that the cell viability of the sample exposed to 8 pulses of 300 ns duration at 20 kV/mm was 84.0%.

The cell viability in all samples, even those tested straight from the culture, cannot be 100% since the cells begin to undergo necrosis immediately after their removal from the growth media and the controlled environment

incubator. It is also important to note that throughout all remaining experiments performed, all measurements of cell necrosis are contrasted with a control viability.

Based on results listed above (see Figure 5) it is reasonable to deduce that the nanosecond pulsed electric field has little to no effect on the viability of cells when used alone (no nanoparticle-cell conjugation). Moreover, a statistical analysis of the data using general linear models (GLM) showed a p-value of 0.4499 when comparing the control vs. 8 pulses at 60ns and 20 kV, indicating that there was no significant difference between the control viability and the nsPEF treated viability. A second statistical analysis of the 300 ns and 20 kV samples gave a p-value of 0.4435, indicating for a second time that there was no significant difference between the control samples and the pulsed samples.

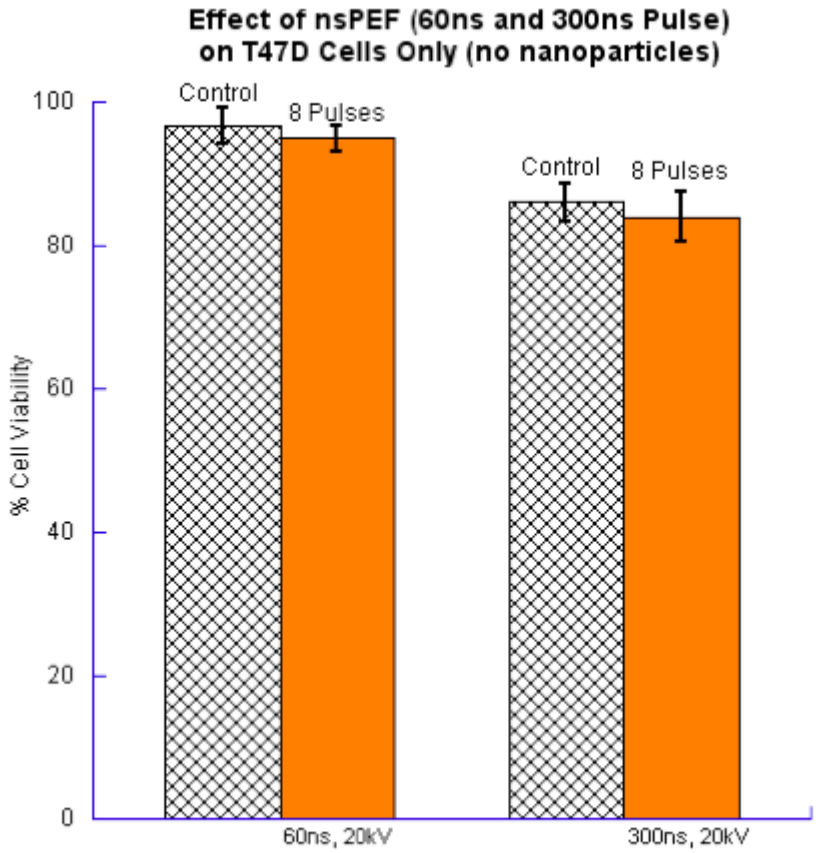

Figure 5: Effect of both 60ns and 300ns nanopulses on **T47D cells only (no nanoparticles present)**. P-value for 60ns experiment is **0.4499**, and for the 300ns experiment is **0.4435**.

3.2 Study Treating T47D Breast Cancer Cells Conjugated to Antibody Specific Iron Oxide Nanoparticles with 60ns Nanopulses

To determine the effect of the 60ns nanosecond pulsed electric field on the T47D cells conjugated with antibody selective iron oxide nanoparticles, the viability was determined at increasing increments of pulses and corre-

spondingly compared with the cell viability of a control sample. This control sample was composed on T47D cells conjugated to antibody selective iron oxide nanoparticles, but was not exposed to the nsPEF treatment.

It was found that after one 60ns pulse at 20 kV/mm, the cell viability was 87.8%. After four pulses under the same parameters, the cell viability was 87.5%. After eight pulses, the cell viability was 35.4%. After 12 pulses, the cell viability was 16.2%. And after 16 pulses the cell viability was 5.5% (Figure 6).

Additionally, a statistical analysis of the data using Analysis of Covariance methods (ANACOVA) resulted in a p-value less than 0.0001 when comparing the effect of pulse number (60ns pulses) on cell viability for the control against the iron-oxide conjugated cells. This p-value indicates that there is a statistically significant difference between the control and the conjugated cells.

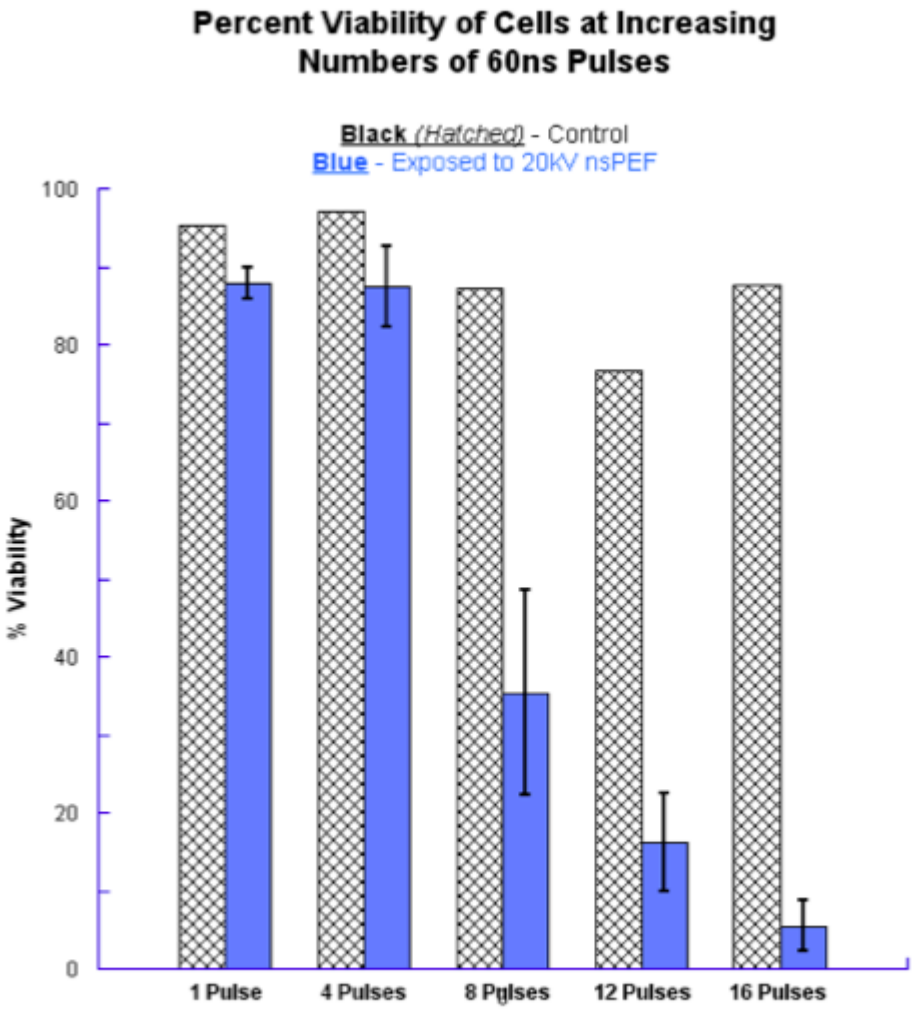

Figure 6: Effect of 60ns nanopulses on T47D cells conjugated to antibody labeled **iron oxide nanoparticles**. The number of pulses increased from 1 to 16 pulses.

To more accurately depict the percentage of cells ablated by the nsPEF treatment, the percent viability of samples exposed to the nanopulses had to be compared and subtracted from each corresponding control. After one 60ns pulse at 20 kV/mm, 7.6% of the T47D cells were ablated. After four pulses, 9.7% of the cells were ablated. After eight pulses, 51.8% were ablated.

After 12 pulses, 60.4% were ablated. And finally, after 16 pulses, 82.2% were ablated (Figure 7).

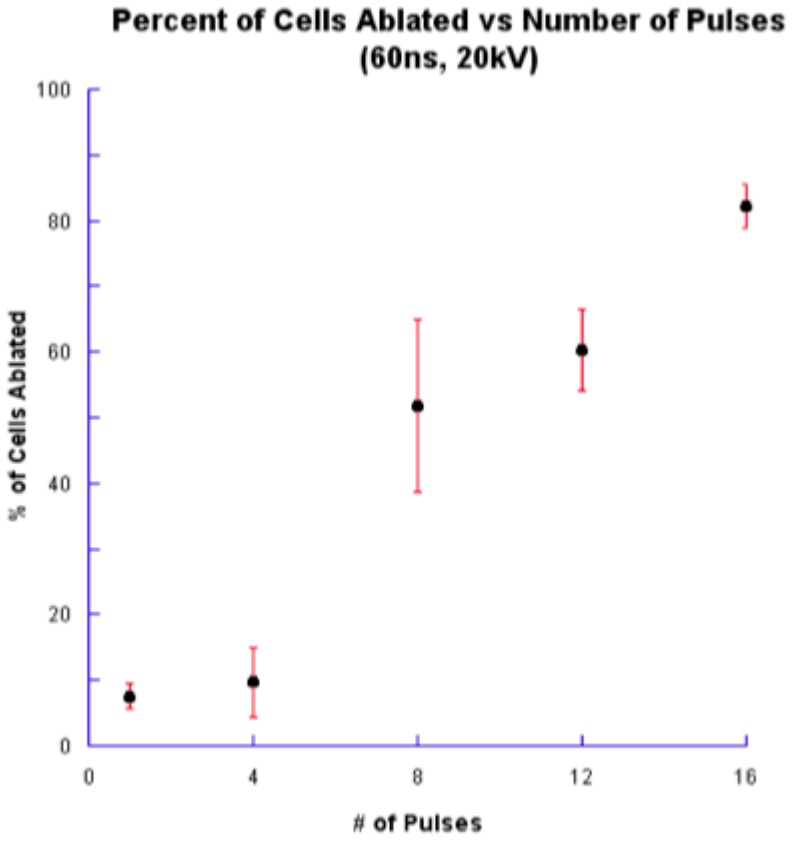

Figure 7: To more clearly show the percentage of cells (conjugated with **iron oxide nanoparticles**) ablated by 60ns pulses, the difference in viability between treated samples and control samples is plotted at increasing numbers of pulses.

3.3 Study Treating T47D Breast Cancer Cells Conjugated to Antibody Specific Iron Oxide Nanoparticles with 300ns Nanopulses

To determine the effect of the 300ns nanosecond pulsed electric field on the T47D cells conjugated with antibody selective iron oxide nanoparticles, the viability was determined at increasing increments of pulses and correspondingly compared with the cell viability of a control sample. This control sample was composed on T47D cells conjugated to antibody selective iron oxide nanoparticles, but was not exposed to the nsPEF treatment.

It was found that after one 300ns pulse at 10 kV/mm, the cell viability was 67.7%. After four pulses under the same parameters, the cell viability was 20.9%. After eight pulses, the cell viability was 10.0%. After 12 pulses, the cell viability was 4.6%. And after 16 pulses the cell viability was 2.6% (Figure 8).

Additionally, a statistical analysis of the data using Analysis of Covariance methods (ANACOVA) resulted in a p-value less than 0.0001 when comparing the effect of pulse number (300ns pulses) on cell viability for the control against the iron-oxide conjugated cells. This p-value indicates that there is a statistically significant difference between the control and the conjugated cells.

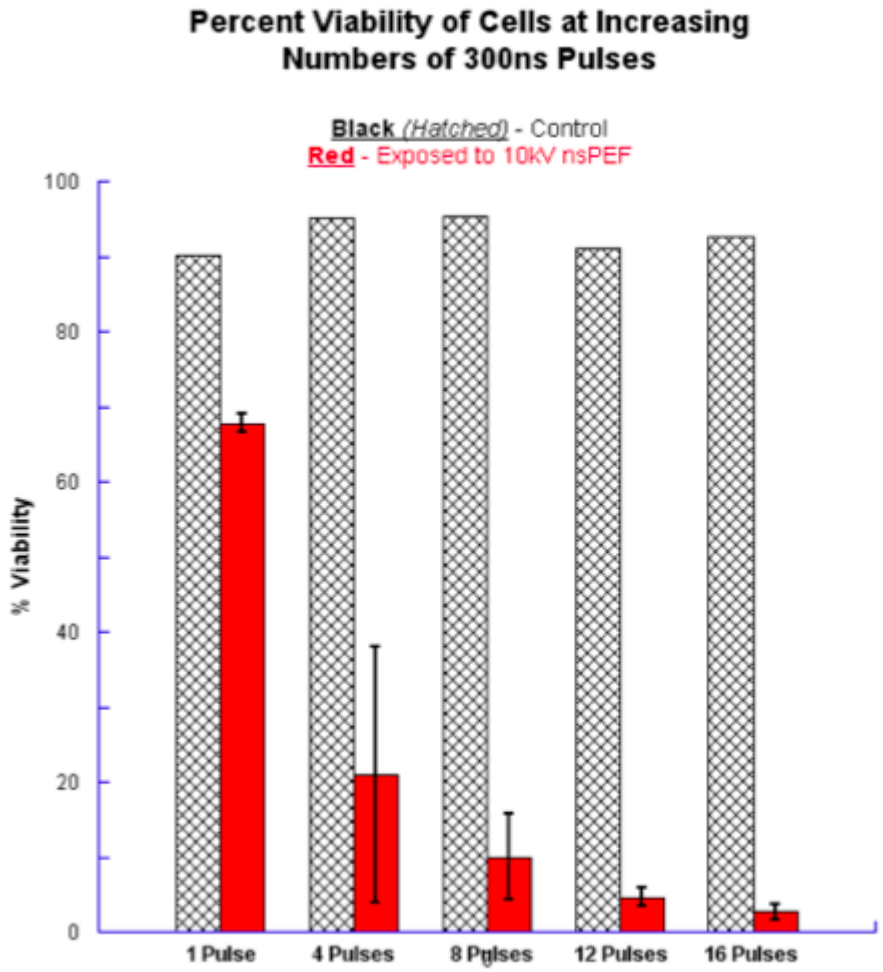

Figure 8: Effect of 300ns nanopulses on T47D cells conjugated to antibody labeled **iron oxide nanoparticles**. The number of pulses increased from 1 to 16 pulses.

To more accurately depict the percentage of cells ablated by the nsPEF treatment, the percent viability of samples exposed to the nanopulses had to be compared and subtracted from each corresponding control. After one 300ns pulse at 10 kV/mm, 22.5% of the T47D cells were ablated. After four pulses, 74.1% of the cells were ablated. After eight pulses, 85.3% were

ablated. After 12 pulses, 86.4% were ablated. And finally, after 16 pulses, 90.0% were ablated (Figure 9).

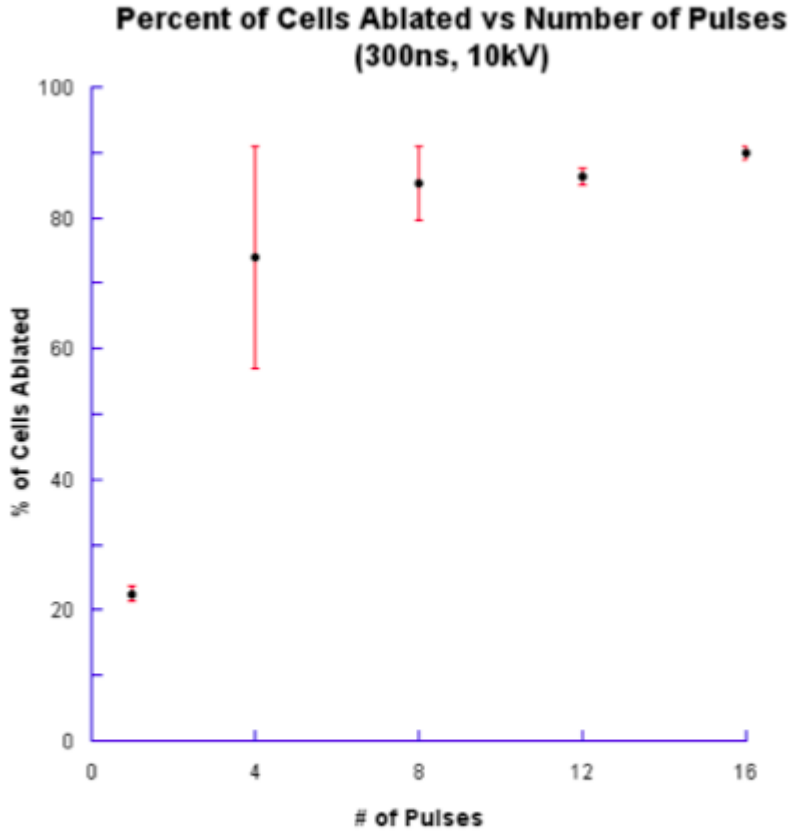

Figure 9: To more clearly show the percentage of cells (conjugated with **iron oxide nanoparticles**) ablated by 300ns pulses, the difference between treated samples and control samples is plotted at increasing numbers of pulses.

3.4 Study Treating T47D Breast Cancer Cells Conjugated to Antibody Specific Gold Nanoparticles with 300ns Nanopulses

To determine the effect of the 300ns nanosecond pulsed electric field on the T47D cells conjugated with antibody selective gold nanoparticles, the viability was determined at increasing increments of pulses and correspondingly compared with the cell viability of a control sample. This control sample was composed of T47D cells conjugated to antibody selective gold nanoparticles, but was not exposed to the nsPEF treatment.

It was found that after one 300ns pulse at 10 kV/mm, the cell viability was 29.1%. After four pulses under the same parameters, the cell viability was 18.2%. After eight pulses, the cell viability was 2.18%. After 12 pulses, the cell viability was 3.35% (Figure 10).

Additionally, a statistical analysis of the data using Analysis of Covariance methods (ANACOVA) resulted in a p-value less than 0.0001 when comparing the effect of pulse number (300ns pulses) on cell viability for the control against the gold nanoparticle conjugated cells. This p-value indicates that there is a statistically significant difference between the control and the conjugated cells.

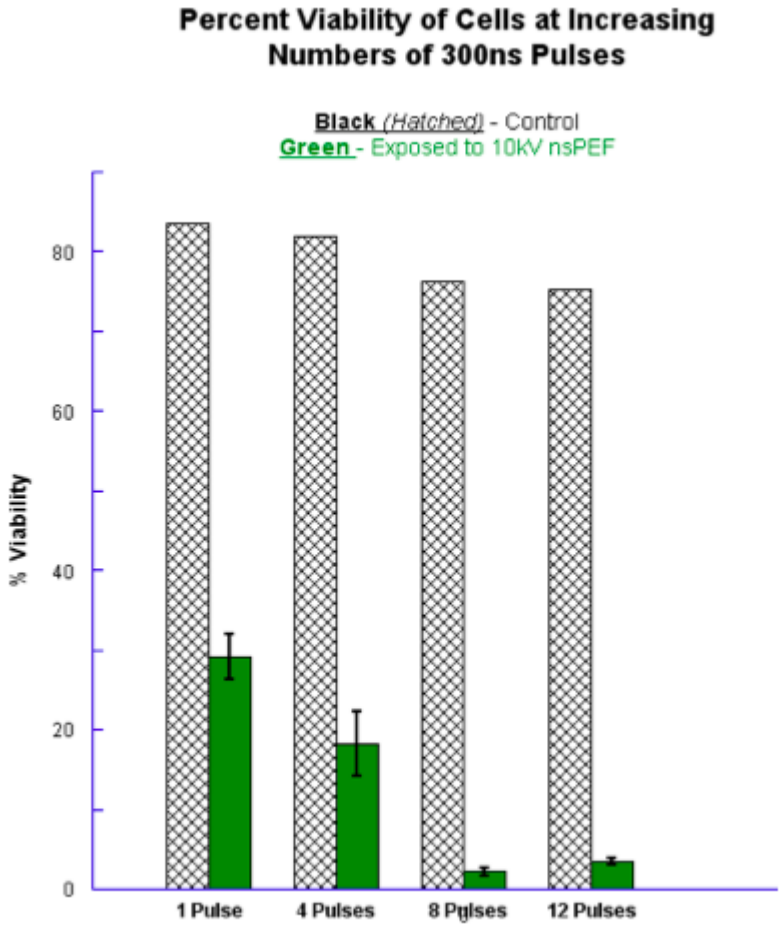

Figure 10: Effect of 300ns nanopulses on T47D cells conjugated to antibody labeled **gold nanoparticles**. The number of pulses increased from 1 to 12 pulses.

To more accurately depict the percentage of cells ablated by the nsPEF treatment, the percent viability of samples exposed to the nanopulses had to be compared and subtracted from each corresponding control. After one 300ns pulse at 10 kV/mm, 54.3% of the T47D cells were ablated. After four pulses, 63.8% of the cells were ablated. After eight pulses, 74.0% were ablated. And after 12 pulses, 71.8% were ablated (Figure 11).

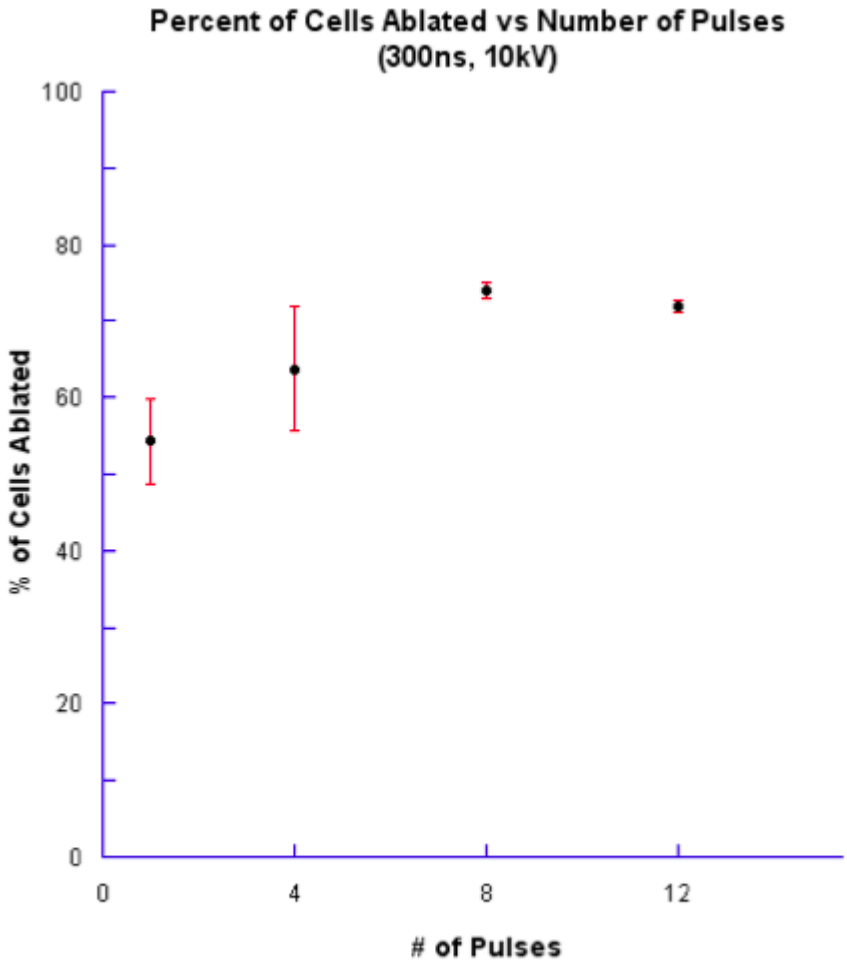

Figure 11: To more clearly show the percentage of cells (conjugated with **gold nanoparticles**) ablated by 300ns pulses, the difference in viability between treated samples and control samples is plotted at increasing numbers of pulses.

3.5 Difficulty Studying The Effect of 60ns Nanopulse Treatment on T47D Cells Conjugated with Gold Nanoparticles

When examining T47D cells conjugated with gold nanoparticles treated with 60ns pulses, we were unable to collect any concrete results. This inconsistency can possibly be attributed to several factors. First, the generation number of the cells cultured during the time of experimentation was very high. It was discovered that the cells being tested upon were of the 40th generation and beyond. Second, the Accumax solution used to achieve some semblance of monodispersion amongst the cells was also significantly aged. Both of these above postulations could explain why the viability of all control samples for every test attempted on T47D cells conjugated with gold nanoparticles and treated with 60ns pulses, were between 59.2% and 75.6%; much lower than those from any other studies. Additionally, in some 60ns, gold nanoparticle experiments, very few, if any cells were ablated by the nanopulse treatments. This could be potentially explained by the aging of the anti-EpCAM antibodies. If the conjugation procedure was faulted by the aging/denaturation of the antibodies, a significant quantity of the nanoparticles may not have been attached to the cells and thus unable to generate ablative heat due to lack of contact with the cells.

4 DISCUSSION

Based on the results shown in Section 3.1, the nanosecond pulsed electric field, at pulse durations of both 60ns and 300ns, is not harmful to the cancer cells alone. The treated samples had very close viabilities to the corresponding control samples (no nsPEF treatment). Showing this result was a critically important step to proceeding with other studies because it demonstrated that any cell necrosis achieved was largely attributed to the heating of the metallic nanoparticles, and not an additional effect of the nsPEF. It can also be concluded from this study that the nsPEF may not have any significant effect on the viability of potentially "healthy cells" should these studies progress to a discrimination study between healthy human cells and cancer cells using this treatment modality.

Given the high percent viability of the control samples (most were greater than 90%) in Sections 3.2 and 3.3, it can be concluded that the iron oxide nanoparticles were non-toxic to the T47D cells. Furthermore, both the 60ns treatment and the 300ns treatment were effective in generating significant percentages of necrosis in the samples at higher numbers of pulses. The 300ns treatment, however, was able to reach ablation percentages above 80% with fewer pulses than the 60ns treatment. This result was to be expected considering the total exposure time of a single 300ns pulse is 5 times longer in duration than a single 60ns pulse. Additionally, it should be noted that the 300ns treatments were conducted at 10 kV/mm, half the voltage of the

60ns treatments. This was done to prevent the cracking and leaking of the sample cuvettes.

In Section 3.4, where gold nanoparticles were used, the control viabilities were around 80%, slightly lower than the studies performed with iron oxide nanoparticles. Some of this difference can be attributed to the aging cell generations, but it is believed that some may also be attributed to a mild toxicity effect of the gold nanoparticles as compared to the polymer-coated iron oxide nanoparticles. The 300ns treatment of cells conjugated to gold nanoparticles appeared to be somewhat successful in generating necrosis in the samples, especially at 8 pulses and higher where over 70% of the cells were ablated. The results of Sections 3.2-3.4 along with the failure to gather data mentioned in Section 3.5 seem to indicate that iron oxide magnetic nanoparticles are much more effective as hyperthermic agents than gold nanoparticles when using this nanopulse treatment.

Moreover, the exact mechanism of cell ablation through this nanoparticle and electric pulse interaction is still unclear. It has been demonstrated that a conducting sphere in a uniform electric field would experience bipolar charging [Hwang, 2011]. The resulting charge and the time required to achieve that charge would depend on both the conductivity and permetivity of the medium (for these studies: 1x PBS). Based on the established conductivity and ion mobility values in water and established equations, it takes approximately 1ns for a metallic nanoparticle to reach its final charge in the electric field [Hwang, 2011; Johnson, 2005; Koneshan, 1998].

Based on these speculations, current induced heating only occurs at the beginning and end of each pulse duration used, making both the 60ns and 300ns durations equally effective. The results of the studies disagree with this conclusion given that the 300ns data showed much more effective treatment. Other interactions may be taking effect during the electric field pulses, or interactions between the nanoparticles themselves may be occurring. A study performed by Van den Bosch et al. (1995) describes attraction and repulsion between two conducting spheres in a parallel electric field. The interaction described in this study could have a significant implication to the nanopulse studies involving iron oxide nanoparticles in that there are often multiple particles present around single cells. Based on the results in Sections 3.3 and 3.4 where 300ns pulse were effective with both materials in treating cells, we can assume that the resultant effect is generated by electric effect at this pulse duration. At 60ns however, only the iron oxide nanoparticles were effective in treating the cells, perhaps indicating that at 60ns, the treatment effect is magnetic as opposed to electric, thus why the gold was ineffective. Additionally, it has been indicated that there may be electric field enhancement in a localized region near the nanoparticles on the order of their radius [Davalos, 2009].

Though a complete simulation determining the effects of the electric field nanopulses on the nanoparticles is outside the scope of these studies, research has been performed that shows the local cell membrane temperature would need to reach 45-50°C before the integrity of the cell membrane would be

compromised [Bischof, 1995; Papahadjopoulos, 1973]. Futhermore, an amplified electric field near the nanoparticles could itself generate poration in the cellular membrane via transient currents. Although the results of Sections 3.2-3.4 demonstrate success of the treatment, further research is required to pinpoint the mechanism of action causing necrosis.

To conclude, the pilot studies described in this document demonstrate that using nsPEF combined with metallic nanoparticles can achieve comparable cells death (upwards of 80 and 90%) to many *in vitro* studies using radiofrequency fields. It is imperative to note that the cumulative exposure time of these radiofrequency studies was at least 30 seconds, and often several minutes; whereas, the results obtained using nanopulses were achieved with a total maximum exposure time of 4.8 μs (sixteen 300ns pulses). Although the short duration times used in the obtained results would not likely translate directly for a tissue study, the time required for these tissue treatments using nanopulses would not likely be six orders of magnitude greater (μs vs. seconds). Furthermore, the eventual goal of this nanopulse research is to develop an *in vivo* technique where functionalized specific nanoparticles can be delivered to a patient and then exposed to nsPEF treatment in a non-invasive technique.

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