THE INVESTIGATION OF CONTROLLED RELEASE MICROCHIPS, NANOPARTICLES, AND siRNA FOR GENE DELIVERY IN TISSUE ENGINEERING APPLICATIONS

A Thesis

by

CHRISTINA CHERN

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2008

Major Subject: Chemical Engineering

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Approved by:

Chair of Committee,	Mariah Hahn
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	James A. Silas
Head of Department,	Michael Pishko

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Major Subject: Chemical Engineering

ABSTRACT

The Investigation of Controlled Release Microchips, Nanoparticles, and siRNA for Gene Delivery in Tissue Engineering Applications. (December 2008)

Christina Chern, B.S., Carnegie Mellon University

Chair of Advisory Committee: Dr. Mariah Hahn

The study of drug delivery for the treatment of illnesses and injuries is an important area of pharmaceutical technology. A relatively new area of drug delivery being explored is gene therapy, which utilizes the idea that genes can be used as an alternative treatment. The exploration of gene delivery brought major advancements in the treatment of cancers and tumors as well as many challenges. In this study, the challenges of maintaining a stable vehicle for delivery, delivering genes into the cells, and the efficacy of the gene delivery vehicle were explored.

Seven co-polymers of 12% (w/v) poly (D, L-lactic glycolide) (PDLG) were used to find a biodegradable polymer composition as an implant that temporarily controls the delivery of the genes. Of the formulations tested, 65/35 DL 3A and 50/50 DLG 4A were observed to show degradation time frames that best fit our purposes.

Also, nanoparticles have been used to aid in the targeting of drugs to desired cells in delivery. One drawback of using nanoparticles is the potential toxic side effects they might cause. Zinc oxide nanoparticles coated with poly (vinyl pyrrolidone) (PVP) used as drug carriers were observed to have an effect on cell viability in previous studies. The

cytotoxic effects of ZnO nanoparticles and PVP have on NIH 3T3 mouse fibroblast cells were investigated to see if there is a direct correlation between the level of PVP and zinc nanoparticles to the amount of cell death. It was found that an increase in concentration of ZnO nanoparticles correlates to a decrease in viability of the cells. It was also noted that the method of cell death is likely to be apoptosis.

To confirm the efficacy of gene therapy through transfection, the transfection of the serum response factor (SRF) gene plasmid DNA and short interfering RNA (siRNA) were investigated. The efficiency of the transfection method were tested for both two-dimensional and three-dimensional transfection of the SRF plasmid and siRNA. Experiments with two-dimensional transfection of the SRF plasmid and siRNA were successful, and transfer of the gene in the three-dimensional environment was observed with promising results with the siRNA.

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In addition, special thanks to Mauricio Baez in Dr. Arul Jaramayan's group and Dany J. Munoz for helping with the cell death detection ELISA assays. I would also like to thank Paul Qu, and Dr. Arul Jaramayan and Dr. Thomas Wood's labs for their help with the SRF transfection experiment.

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1. INTRODUCTION: DRUG DELIVERY

1.1 The Importance of Drug Delivery

Drug delivery is an important aspect of pharmaceutical technology and tissue engineering in treating illnesses and injuries with substances that can potentially improve the conditions of patients. Tissue engineering involves the development of new materials that encourage cell infiltration and proliferation to treat or replace damaged tissue. Controlled, targeted release of drugs brings therapeutic effects for the engineered tissues in applications such as wound healing, anti-inflammatory treatment near implants, and anti-tumor therapies. Drugs can be delivered in many forms such as solids, liquids, and aerosols. They can also be delivered in conjunction with a drug carrier to assure targeted drug delivery effectiveness by preventing the drug from being absorbed or degraded on its way to the target site or by directing the drug to its target site. Effectively delivering the drug to the target site and have the drug maintain its activity is harder than it seems as the human body has many natural barriers that prevent foreign drug molecules from penetrating and passing through such as the blood-brain barrier and the stratum corneum of the skin[1, 2]. Phagocytes may also take up the drugs and deliver it to undesired destinations in the body[3].

Gene therapy utilizes the concept that a gene can be inserted into the cells of patients through transfection to either replace or deactivates a mutated or damaged gene

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that is causing the disease. The gene can also be a gene that was not present before that is introduced to help fight the disease. This method is attractive for diseases with no current cures, such as hereditary disorders, cancers, and certain viral infections[4]. In theory, gene therapy can be done simply though intravenous injection of siRNA or plasmid DNA at the site. The cell takes the gene into the nucleus through endocytosis as shown in Figure 1 with a lipid-based non-viral vector[5].

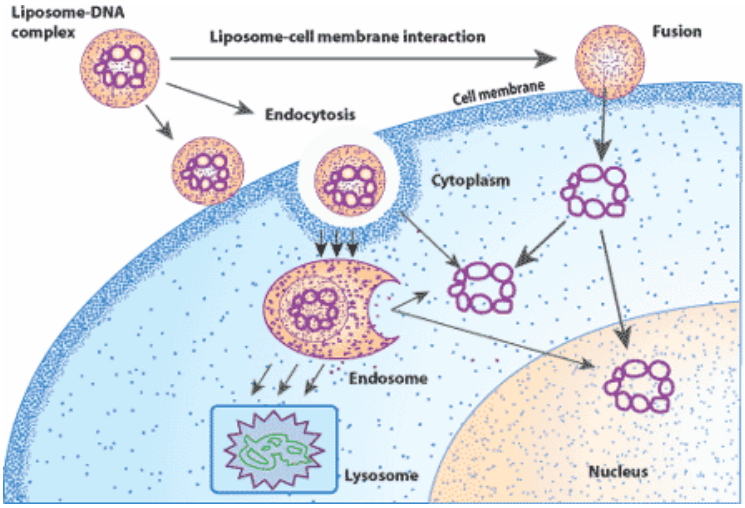


Figure 1. Schematic of How Gene Transfer Takes Place in a Cell[6].

1.2 Current Methods and Approaches

Drug delivery methods currently used include injections, trans-dermal diffusion, ingestion, inhalation, and timed release implantation[7]. The method of delivery depends on the molecule size, location of the target site, and the purpose of the drug. The advantages of trans-dermal diffusion are that it provides an alternative method of drug

delivery when other methods are not ideal, the skin provides a relatively large and readily accessible surface area, and a path-like device to the skin surface is non-invasive but at the same time it allows continuous intervention. A major drawback of trans-dermal diffusion is that bigger molecules cannot be absorbed through the skin and will have to be delivered via another method since they will not be able to get through the tortuous stratum corneum layer of the skin. Proteins, peptides, and oligonucleotides are also difficult to deliver through trans-dermal diffusion due to their half-lives, but they are still desirable sources of drugs due to their highly specific and potent nature compared to other forms of drugs[2].

Injection is also an effective method if possible, but there is pain involved for the patient as well as problems with maintaining a therapeutic effect for drugs with short half-lives and the fact that it is an invasive procedure. Almost all types of drugs can be administered through injection such as nanoparticles, proteins, antibodies, and viral particles.

Inhalation utilizes the large surface area of the alveoli of the lungs that can absorb drugs, which increases the speed of the uptake of drugs, the relatively thin epithelial barrier, extensive vascularization and low proteolytic activity in the alveolar space of the lungs. Smaller molecules can also be delivered through this method due to the thin epithelial barrier that it has to overcome to be absorbed into the system. Aerosol drug delivery is a non-invasive delivery method that also prevents the potential for poor absorption and/or high metabolism in the gastrointestinal tract and first-pass losses in the liver of patients that are seen in oral delivery methods[3].

Timed-release implantations have also been used in many applications such as for the delivery of parathyroid hormone (PTH) for patients with osteoporosis and stent implants. In this study, gene delivery for timed-release implantation applications was explored.

These different types of drug delivery methods are desirable as long as certain criteria are met in order for them to be suitable drug delivery systems. The material used as the carrier should be biocompatible (i.e., no toxic byproducts should form when it is inside the body), limited premature release of the drug, cell type or tissue specificity and site directing ability, and controlled release of drug molecules with a proper rate of release to achieve an effective local concentration. These requirements should be met as close as possible to reduce inflammatory response to the foreign particles or systems as well as reduced efficiency of the drug that is applied[8].

2. GENE DELIVERY IN TISSUE ENGINEERING APPLICATIONS

2.1 Controlled Release Microchips

The effectiveness of the drug is affected by how the drug is released into the system. Immediate release drug delivery is not beneficial to patients with certain diseases or disorders such as asthma, arthritis, and gastric ulcer due to the nature of the human body following a rhythmic pattern, so treatments at certain times are more effective than if the drug dosage was administered all at once. Pulsatile drug administration allows for controlled and predetermined application of drugs over a period of time and helps maintain a therapeutically effective level of the drug within the patient's system. Pulsatile drug release is another area of research that has been gaining attention due to the different materials that can be used to achieve this effect when administered. These delivery systems are either stimulus-induced or self-regulated release systems. Stimulus-induced delivery systems are stimulated by temperature, pH, light, enzyme or electric and magnetic fields. There have been promising results from stimulus-induced delivery systems, but they are usually made of non-biodegradable material in which the host body will elicit an immune rejection towards the implant. Self-regulated delivery systems are usually erodible or biodegradable polymers with an encapsulated drug. The release times are often times varied by the thickness and structure of the encapsulation material. While this delivery system utilizes biodegradable and biocompatible materials as the vehicle of delivery, it is difficult to fabricate the different thicknesses for different release times on a small scale, which leads to inconsistent results seen in several reports[9]. Doctors use the knowledge of the

behavior of the drug and its purpose to determine dosages, how often to administer the drug, and how the drug should be delivered to the patient.

An example of a timed-release implantation system would be stent implants with a coated layer of drugs. Stent implants save the lives of many patients with cardiovascular diseases, but the implant poses a problem as a foreign object in the body. It is also associated with an excessive proliferation of vascular smooth muscle cells, extracellular matrix synthesis, thrombosis, and chronic inflammation. One solution to these problems is to fabricate the stent with a coated polymeric layer in which therapeutic agents are released gradually after implantation to alleviate the biological responses[10]. This method of drug delivery has also been used in other applications for insulin delivery or parathyroid hormone (PTH) delivery where it has been shown to stimulate bone formation through improving bone micro-architecture, mineral density, and strength for patients with osteoporosis[11]. In addition to using pulsatile delivery systems for timed release of drugs over an extensive period of time, the technology can also be used for other applications where the drug needs to be applied locally, is not easily absorbed or has an extensive first pass metabolism.

A self-regulated release drug delivery system example would be the microchip multi-dose drug release device Grayson et al[12] developed, which is explored in this work for its application as a stable means of delivering genes as drugs. For this device, degradable polymers are used as the cover on the reservoir openings as well as the microchip material as seen in Figure 2.

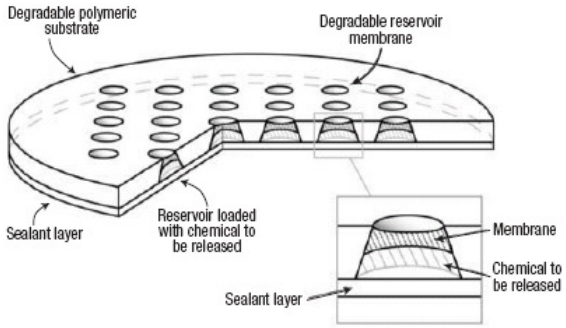


Figure 2. Schematic of the Self-Regulated Microchip Multi-Dose Device[12].

The premise of the design is that each reservoir is sealed with polymers with different properties for different degradation times[12]. These varying properties could be the molecular mass, material, thickness or composition of the membrane. In this work, the composition of the PDLG copolymer will be varied to identify formulations that show a degradation time of around two weeks for our purposes. The use of an implantable biodegradable device will allow the release of the gene into body over a period of time, which saves the patient from having to visit the hospital regularly for injections, surgeries, and hospital visits for drug administrations.

2.1.1 Materials

In this work, the degradation times of the co-polymers with compositions of 12% (w/v) of each 65/35 DL 3A, 50/50 DLG 2.5A, 50/50 DLG 4A, 50/50 DLG 5A, 75/25 DLG 1A, 75/25 DLG 2A, and 85/15 DLG 1A, where the convention of nomenclature is based on the percentage of each lactide and glycolide concentration in the polymer and the number following the co-polymer acronym denotes the chain length, were observed. The polymers were dissolved in 99% 1, 1, 1, 3, 3, 3 – Hexafluoro-2-propanol and were

monitored through microscopy to identify polymer compositions that have an approximate two week degradation time frame. The polymers degraded through hydrolysis from the water present in the media and likewise in the body. Polymer degradation is largely dependent on its rheology and morphology. The degradation time of a polymer depends on its hydrophilicity, glass transition temperature (Tg), crystallinity, molecular weight, and porosity. The polymer used in this study for the timed release experiment is PDLG, which is a co-polymer of lactide and glygolide. Lactide and glycolide are similar in structure with the exception of a methyl side group on lactide (Figure 3), which makes the polymer less crystalline and more amorphous[13]. Polyglycolide has a Tg of between 35-40 ^{o}C and polylactide has a Tg of between 50-80 ^{o}C [14, 15] and polylactide has a higher molecular weight due to the extra pendant group.

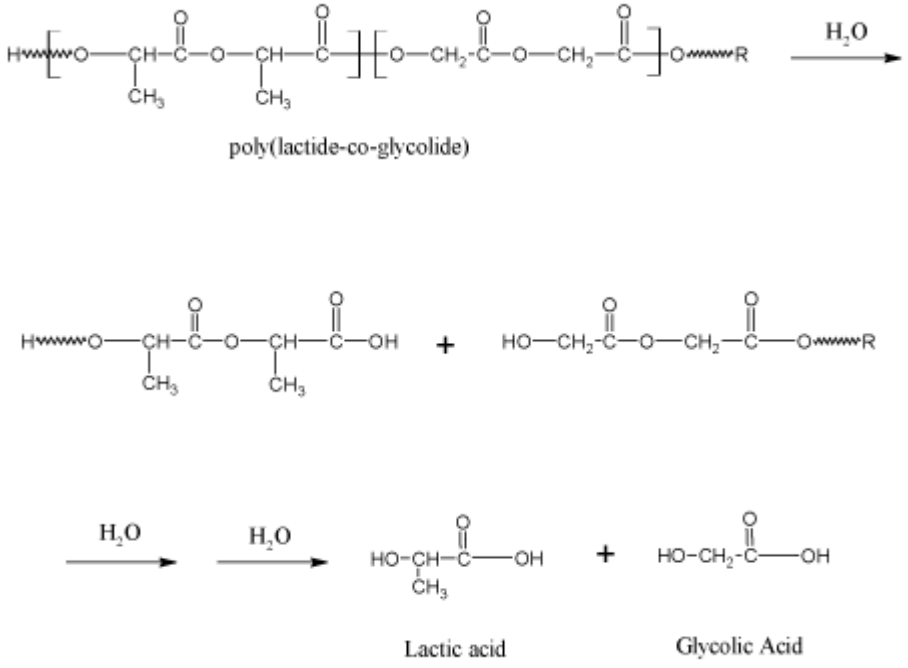


Figure 3. Schematic of the Degradation of PDLG[16].

By looking at the polymer structure and properties it can be theorized that the polymer with a higher lactide content and longer chain will have a longer degradation time due to the fact that it has a higher Tg and is less hydrophilic even though it is less crystalline in structure[13]. We hypothesized that 50/50 DLG 5A and 50/50 DLG 4A will be the formulations that best fits our experiment purposes due to the higher concentration of lactide and longer chain of the polymers.

2.1.2 Methods

2.1.2.1 Making of Microchips

Microchips for pulsatile drug release were created by using a Teflon mold with four peaks. The microchips were made with 1g of 100 DL 4.5A (Lakeshore Biomaterials, Birmingham, AL). The dry polymer pellets were melted on a Carver press at 180 ^{o}F on top of a flat Teflon piece and approximately 13,500 lbs of force was applied to the melted polymer with the Teflon mold on top to make the indentation of reservoirs to hold the drugs. The sandwiched piece was cooled with running water and pulled apart from the Teflon pieces. It was then sanded down on both sides by hand for a smooth, flat surface with a 400 grit superfine sandpaper (Norton, Sanborn, NY). The microchips were sanded down until the opening was approximately 0.75 mm in diameter.

2.1.2.2 Making of Polymer Capsules

Dry polymer pellets of 65/35 DL 3A, 50/50 DLG 2.5A, 50/50 DLG 4A, 50/50 DLG 5A, 75/25 DLG 1A, 75/25 DLG 2A, and 85/15 DLG 1A (Lakeshore Biomaterials,

Birmingham, AL) were dissolved in 99% 1, 1, 1, 3, 3, 3 – Hexafluoro-2-propanol (Sigma-Aldrich, St. Louis, MO) solution with a 12% (w/v) concentration. A table of the properties of the polymers is presented[16]:

Table 1. Polymer Properties

Polymer Name	**Intrinsic Viscosity**	**MW (kDa)**
6535 DL 3A	0.37	45
5050 DLG 2.5A	0.25	28
5050 DLG 4A	0.39	
5050 DLG 5A	0.52	
7525 DLG 1A	0.12	6.6
7525 DL PLG 2A	0.17	14
8515 DLG 1A	0.14	8.1

The desired thickness of the polymer capsule is 150 μm, which through calculations yields about 2.5 μL of polymer solution each well. The microchips were dried overnight in an open hood and placed in a vacuum oven overnight with approximately 27 inHg of pressure in order to remove the solvent from the polymer capsules. The microchips were initially sealed by applying tape to the back side. Later experiments was sealed by using a piece of 0.75g of melted 100 DL 4.5A as the backing that was further sealed with 30% (w/v) 100 DL 4.5A dissolved in 99% 1, 1, 1, 3, 3, 3 – Hexafluoro-2-propanol. The disc was dried overnight in an open hood and placed in a vacuum oven overnight with approximately 27 inHg of pressure. The microchips were then sterilized through one hour of exposure to UV light in the hood on each side and placed in a tweleve well plate (B. D. Falcon, Franklin Lakes, NJ) with cell culture media (DMEM + 10% DFBS + PS).

2.1.2.3 Monitoring Degradation

The microchips were placed in two mL of phosphate buffered saline (PBS) (Hyclone, Logan, UT) or culture media and incubated at 37˚C/5% CO_2. The PBS or culture media was changed every two days due to possible side effects the acidic degradation products created through hydrolysis might have on the non-degraded polymers[16]. The openings of the wells were observed daily with a microscope to determine if the wells were opening.

2.1.3 Results

Initial testing of the polymer compositions showed that when the polymers were incubated in PBS, 50/50 DLG 5A was observed to be the only formulation that have a degradation time of around two weeks compared to the others, which was hypothesized earlier. However, when the polymers were incubated in cell culture media it was observed that 50/50 DLG 4A and 65/35 DL 3A were the two formulations that appear to have a degradation time of around two weeks. Figure 4 shows pictures of representative polymer capsule coverings degrading through time.

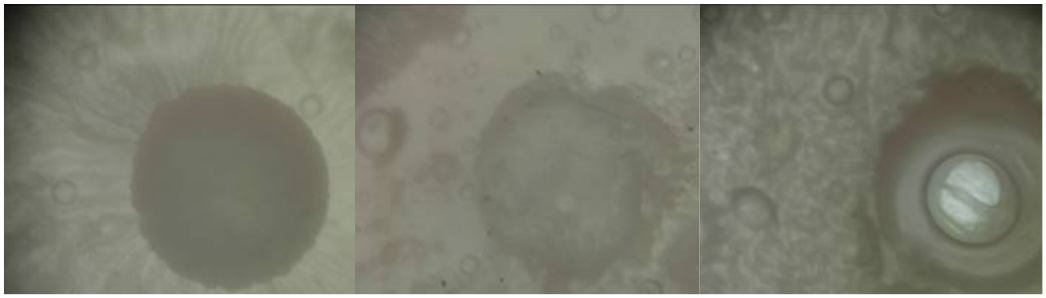


Figure 4. Time Lapse of Degrading Polymer Coverings.

The observations of the degradation times of the two polymer formulations were then repeated to confirm the observations. New discs were made of 12% 65/35 DL 3A and 12% 50/50 DLG 4A with 100 DL 4.5A as the backing and sealed with 30% 100/0 DL 4.5A dissolved in 99% 1, 1, 1, 3, 3, 3 – Hexafluoro-2-propanol. It was observed that 12% 65/35 DL 3A degraded significantly around two weeks, but 12% 50/50 DLG 4A appears to have released in the first four days. Fresh materials were used for the third time where 12% 65/35 DL 3A, 12% 50/50 DLG 4A, and 12% 50/50 DLG 5A were made in the same manner as the previous experiment. 50/50 DLG 5A was included because it should have the longest degradation time due to its polymer properties, and we wanted to see if a fresh batch will yield different results from before. 12% 50/50 DLG 4A was observed to have opened wells after nine days whereas 12% 65/35 DL 3A still exhibited a two week degradation time frame. 12% 50/50 DLG 5A was observed to have degraded within a week.

2.2 The Use of Nanoparticles in Drug Delivery

Nanoparticles are defined as particles less than 100 nm in diameter. It was observed that the properties of known materials changed by decreasing the size to the nanoscale range, thus, creating opportunities for more efficient and versatile uses of these materials[17]. Various methods of drug delivery have been employed throughout the past in pharmaceutical technology including the use of colloidal delivery systems with liposomes, micelles, and nanoparticles. These systems arose because of the need of a drug carrier that would prevent the encapsulated drug from being degraded or provide a

way to target the drug to a specific site in order to limit contact with the surrounding tissue with the drug and maximizing the amount of drug delivered to the desired site.

Nanoparticles are beneficial in drug delivery due to the size of the drugs that can be used as well as their flexibility and convenience in methods of formulation and delivery. They do not settle easily, dissolve quickly, and have a high affinity for adherence to biological surfaces. These particles can be administered through a variety of methods such as oral, pulmonary, parenteral, intraocular or transdermal drug delivery systems depending on the effectiveness of each for the target site. Usually, site-specific delivery to target organs is administered through parenteral injection and delivery to the respiratory tract is administered through inhalation of aerosols[18]. Another advantage of nanoparticles is that they are able to change their surface properties and have large surface areas that are not characteristic of other delivery systems. Nanoparticles are also ideal for delivering drugs to a target tumor site for chemotherapy since it can be selectively delivered in large quantities to the tumor site while at the same time preventing the cause of some of the toxicities seen in solvent-based formulations[19].

Different nanoparticles with various methods of delivery have been used in different applications. One example is the use of nanoparticles for delivering drugs to the lungs in the treatment of lung diseases due to the fact that a higher concentration of drugs can be delivered and the particles are able to retain in the lungs and have a prolonged drug release when nanoparticles are used as a drug carrier. It was found that intravenous injection of the nanoparticles to the lungs was not very effective as most accumulated in the reticuloendothelial system, but aerosol delivery would be the best

method due to the many advantages[5]. Magnetic nanoparticles that are made up of a magnetic core (iron oxide, for example) with a biocompatible polymeric outside covering (dextran, for example) have also been studied as drug carriers. These have been used to target tumor sites for chemotherapy in which they can be easily retained at the desired site with an applied magnetic field[20].

However, using nanoparticles in conjunction with the drug to be delivered or by itself as a drug can have its drawbacks. Nanoparticles can travel to other sites in the body other than its target site and cause adverse side effects in remote organs or tissues[5]. The changing of the physical and chemical properties of these materials may lead to a different biological property, which is why the toxicity of nanoparticles is an area of study that has been gaining attention[17].

Zinc oxide nanoparticles coated with PVP were used in this study where the cytotoxicity of these particles to NIH 3T3 cells is assessed before determining the feasibility of its application for use in gene delivery. PVP is used to prevent aggregation of the zinc oxide nanoparticles and zinc oxide was chosen since it is a commonly used nanoparticle in personal care products[21, 22]. It is proposed by Dr. H. J. Sue's group (Mechanical Engineering, Texas A&M University) that the zinc ions interact with the phosphates in the cell culture medium to form zinc phosphate crystals that penetrate cell walls and cause the cells to rupture. The group's theory is that an increase in the amount of PVP used will prevent zinc oxide nanoparticle aggregation, which should result in a decrease in cytotoxicity due to the smaller particles formed. Once the mechanism of cell death is understood, the nanoparticles can be modified to be more biocompatible and

have less biological side effects for its application in biological systems such as for the treatment of prostate cancer. The assessment of cytotoxicity is done in three parts where the metabolic activity, Lactate Dehydrogenase activity (LDH), and amount of DNA fragmentation of the cells are quantified along with the observation of cell viability under the microscope. A new control where PVP without nanoparticles was introduced in this study to investigate the effects of PVP alone on the cells. This study is a novel approach in studying the cytotoxic effects of zinc oxide nanoparticles on cells with the possibility of the presence of phosphates being the reason for the cause of death.

2.2.1 Materials

Preliminary assessment of cytotoxicity will be done in four parts where the metabolic activity, LDH activity, and amount of DNA fragmentation of the cells are quantified along with the observation of cell viability under the microscope. The assays will be carried out after the NIH 3T3 cells have been treated with the various nanoparticle concentrations overnight. The NIH 3T3 cells will be tested with two different types of cell culture media: media with phosphates and media without phosphates. A control where PVP without nanoparticles was introduced in this study to see the effects PVP alone has on the cells. This work is a novel approach to determine the cytotoxicity of the nanoparticles based on the hypothesis that the presence of phosphates played a part in the process. NIH 3T3 cells were subcultured at 37˚C/5% CO_2 in DMEM supplemented with 10% DFBS, 1% penicillin streptomycin (PS) (Cellgro, Herndon, VA). PS is added as an antibiotic for the cells to prevent

contamination and growth of bacteria in the culture[23]. The experiments were conducted using cells at passages 5-8.

2.2.2 Methods

2.2.2.1 Preparation of Cells

NIH 3T3 mouse fibroblast cells[24] were seeded at a density of 25,000 cells/cm^2 in a ninety-six well cell culture cluster plate (Costar; Corning, NY) with two different cell culture media in a final volume of 50 μL/well. One set of cells were subcultured at 37˚C/5% CO_2 in DMEM supplemented with 10% DFBS and 1% PS overnight. The other set of cells were subcultured at 37˚C/5% CO_2 in DMEM without phosphates supplemented with 10% dialyzed DFBS and 1% penicillin streptomycin glutamine (PSG) (29.2 mg/mL L-glutamine; Cellgro, Herndon, VA) overnight. The confluence of the cells was checked under the microscope to be at least 90% confluent before carrying out the experiment.

2.2.2.2 Preparation of Nanoparticle Solutions

Nanoparticle solutions were provided by Dr. H. J. Sue's group in PVP:ZnO concentration ratios of 300:1, 200:1, and 100:1 with a concentration of 0.5 mg/mL ZnO dissolved in deionized (DI) water. ZnO concentrations of 50 μg/mL, 40 μg/mL, 30 μg/mL, 20 μg/mL, 10 μg/mL, and 5 μg/mL were tested on the cells for each ratio of PVP:ZnO. 50 μg/mL and 5 μg/mL of pure PVP (provided by Dr. Mike Bevan's group) dissolved in double DI water were also tested on the cells for each ratio. For the control, no cells were used for the zinc oxide concentrations of 50 μg/mL, 30 μg/mL, and 10

μg/mL as well as the 50 μg/mL and 5 μg/mL of pure PVP for each ratio. The different concentrations of zinc oxide solutions were diluted with double DI water and four times concentration of media (DMEM + 10% DFBS + 1% PS and DMEM without phosphates + 10% dialyzed DFBS + 1% PSG) to reduce the amount of final volume since no media was removed during the experiment to prevent disturbance of the cells.

2.2.2.3 Cell Metabolic Assay

120 μL of nanoparticle solution was added to each well with cells. 200 μL of nanoparticle solution was added to each control well without cells. The plates are incubated overnight with the nanoparticle solutions. Vybrant™ Cell Metabolic Assay Kit (V-23110) (Molecular Probes, Eugene, OR) was used to assess the metabolic activity of the cells after twenty-four hours of incubation with the nanoparticles. This assay measures the amount of red-fluorescent resorufin present in the samples, which is the resulting product of reduced nonfluorescent resazurin by the enzyme glucose-6-phosphate dehydrogenase present in viable cells. 50 μL/well of 10μM C_{12}-resazurin stock solution was used in the experiment and the plate was incubated with the reagent at 37˚C/5% CO_2 for three hours before making readings. 100 μL was taken from each well and the fluorescence at 530 nm absorbance and 590 nm emission was measured with a Multi-Detection Microplate Reader with a sensitivity of thirty (BioTek Instruments, Winooski, VT).

2.2.2.4 Cytotoxicity Detection with Lactate Dehydrogenase (LDH) Activity Assay

120 μL of each media was used to seed the cells in the initial stage of the experiment instead of 50 μL. The same convention is used here where one set used DMEM with phosphates and another set used DMEM without phosphates. After letting the cells grow to confluence overnight, 120 μL of the nanoparticle solution was added to each well with cells. 240 μL of nanoparticle solution was added to each control well without cells. In addition to the "low control" where cells received media without nanoparticles in the solution there was one more control used for this assay. A "high control" was used where the cells were treated just like the low control but were lysed just before the assay for maximum cell death. LDH is present inside the cell as well as in the cytosol, so lysing the cells would allow the maximum amount of LDH measured. The amount of cell death was measured with the cytotoxicity detection kit^{PLUS} (LDH) (Roche, Mannheim, Germany). This assay is a non-radioactive colorimetric assay that measures the amount of cell death and cell lysis based on the LDH activity released from the cytosol of damaged cells. The reagents were prepared according to the kit and 200 μL of each sample was taken. The samples were centrifuged for five minutes to reduce the amount of cell debris in the solution and 170 μL of each sample was transferred into a new tube. 100 μL of each sample was used for the assay. The remaining solution was stored in -20 ^{o}C for the cell death detection ELISA assay. The samples were incubated with the reagent for fifteen minutes before applying the stop solution. The absorbances of the samples were read with a Multi-Detection Microplate Reader at 490 nm

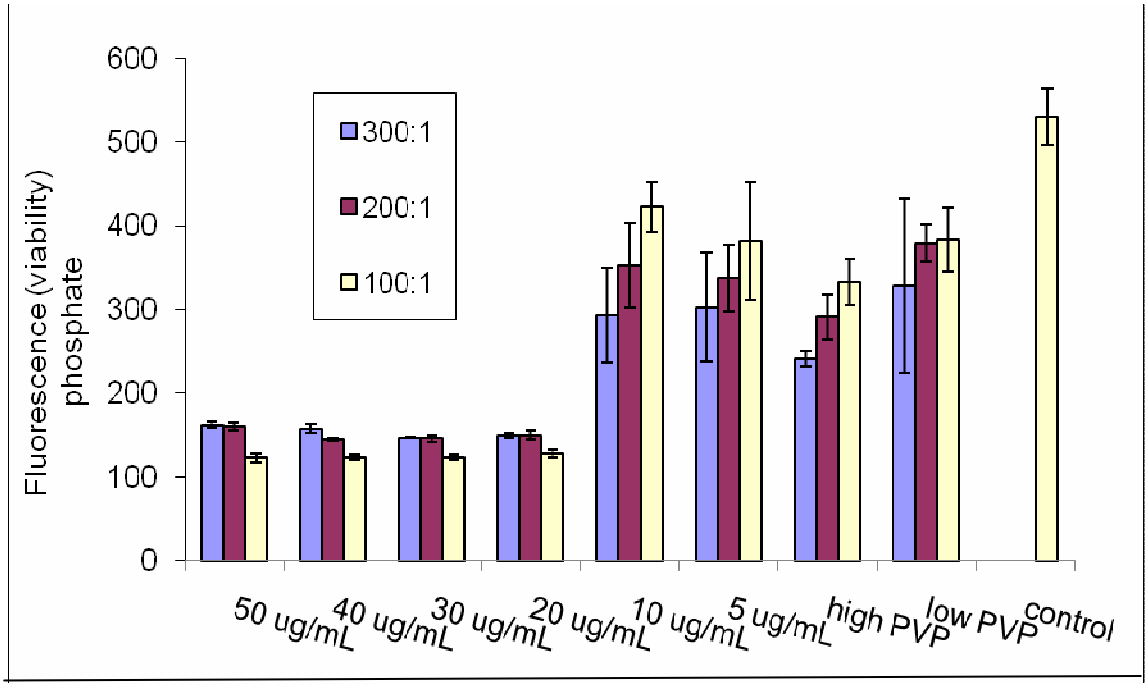
immediately after applying the stop solution. The measured absorbances were plotted against the metabolic measurements for viability/cell death comparisons.

2.2.2.5 Cell Death Detection ELISA Assay

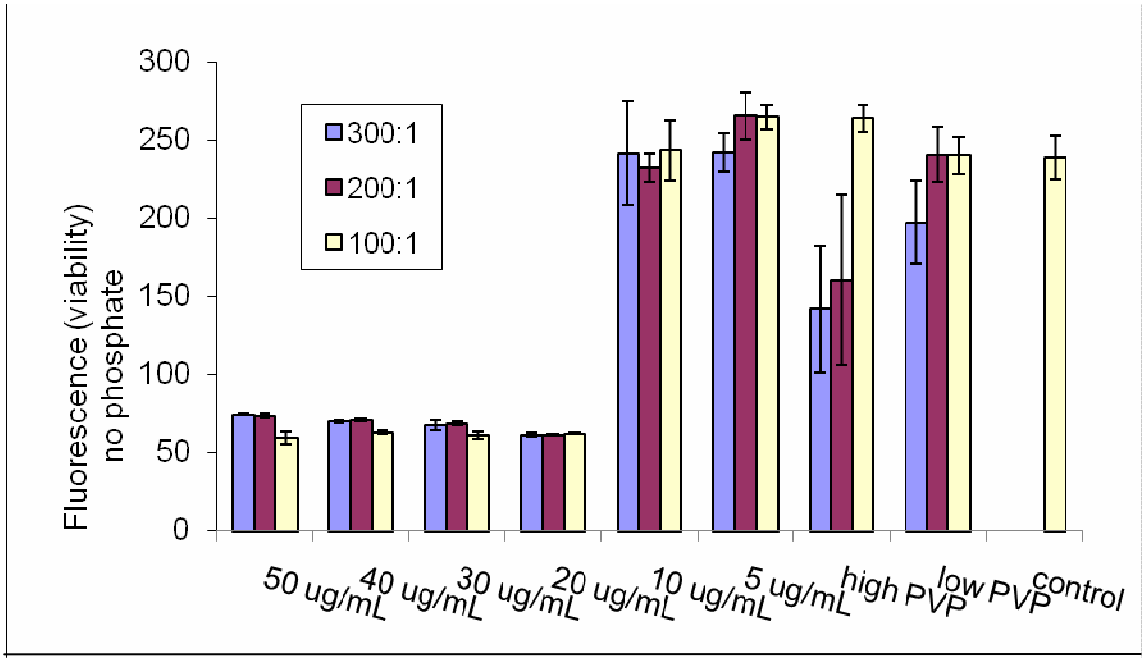
The amount of cell death was also quantified through the utilization of the Cell Death Detection ELISA kit (Roche, Mannheim, Germany). This kit is a photometric enzyme immunoassay that determines the quantitative and qualitative cytoplasmic histone-associated DNA fragments after induced cell death. The absorbances of the samples were read with a Multi-Detection Microplate Reader at 405 nm.

2.2.3 Results

It was observed that there is a significant drop in cell viability between 10 and 20 µg/mL of zinc oxide concentrations for both of the types of cell culture medium used in all ratios of ZnO : PVP concentrations. The results from the Vybrant™ Cell Metabolic Assay Kit fluorescent readings showed that for both of the cell culture medium types the viability of the cells dropped drastically from 10 to 20 µg/mL of zinc oxide concentrations. It can be seen in Figure 5 that the trend is the same in both culture types where an increase in zinc oxide concentration showed lower cell viability. It was also noted that there seemed to be a significant difference between the viability of the treated cells in media with phosphate and the control whereas minimal difference was seen with the treated cells in media without phosphate when compared to the control.

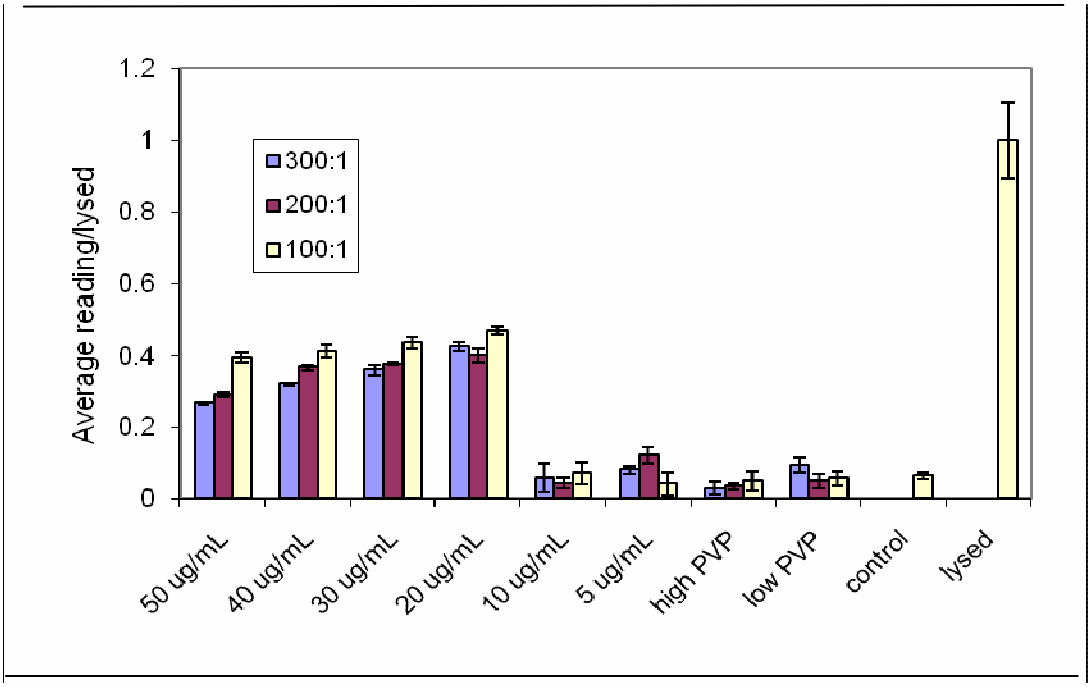


(a)

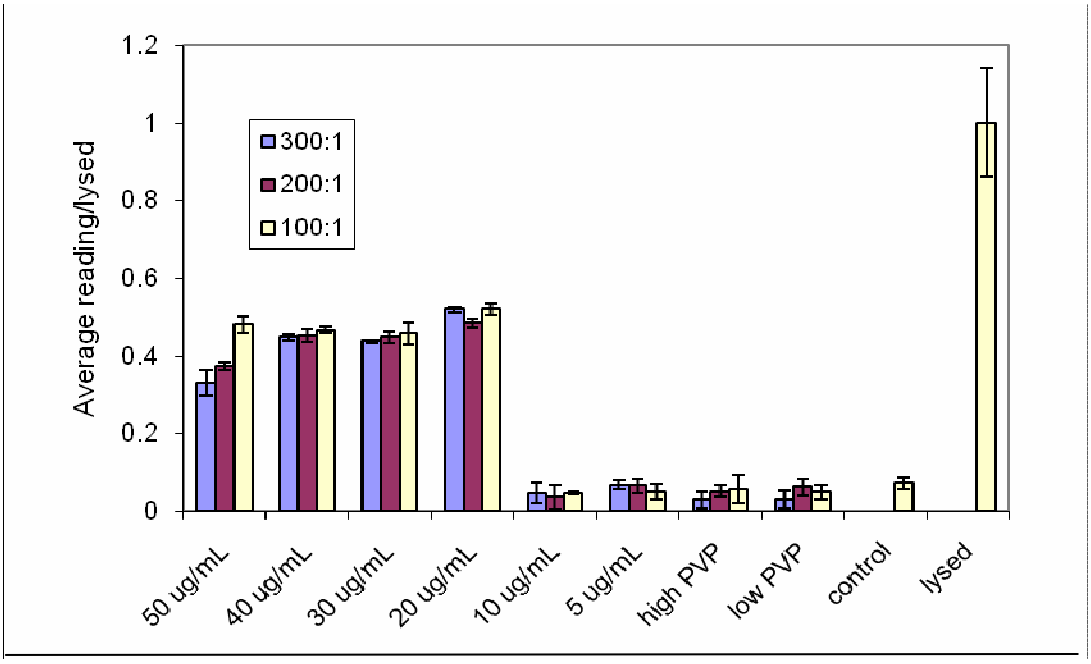


(b)

Figure 5. Cell Metabolic Assay: (a) DMEM with Phosphate after 3 Hours of Incubation with Reagent. (b) DMEM without Phosphate after 3 Hours of Incubation with Reagent.



(a)



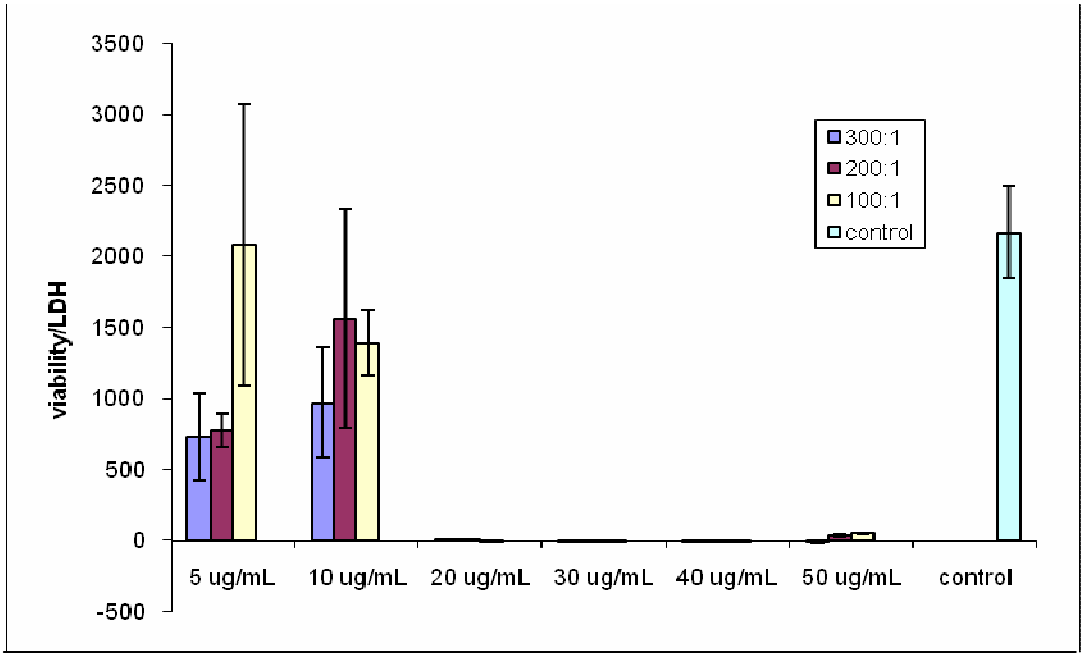
(b)

Figure 6. LDH Assay: (a) DMEM with Phosphate after 15 Minutes of Incubation with Reagent. (b) DMEM without Phosphate after 15 Minutes of Incubation with Reagent.

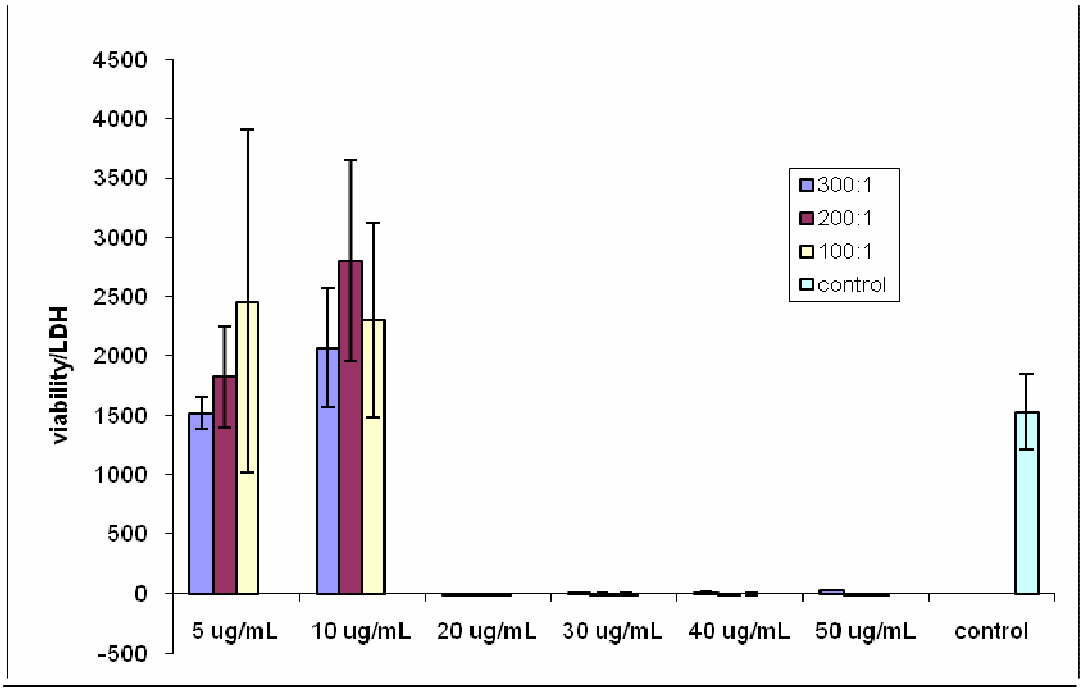
The data also showed that for samples that were treated with just PVP the trend seemed to be the opposite where an increase in PVP concentration reduced cell viability while for cells treated with nanoparticles it was seen an increase in PVP concentration increased cell viability.

For the LDH assay similar trends were observed in the samples where a significant number of cell death was seen between 50 and 20 µg/mL zinc oxide concentration. It can be seen in Figure 6 that a decrease in ZnO : PVP ratio in conjunction with a decrease in zinc oxide concentration increases the amount of cell death. The samples from media without phosphate have generally a higher number of cell death than samples from media with phosphates. The effects for 10 and 5 µg/mL zinc oxide concentration could not be assessed due to the high numbers from the other samples. The viability/LDH results (Figure 7) showed that 10 µg/mL zinc oxide has a higher viability than 5 µg/mL ZnO for all ratios of ZnO to PVP.

For the cell death detection ELISA (Figure 8) it can be seen that for the samples that were in media without phosphate the absorbance was roughly four times as much in the higher zinc oxide concentrations than 10 µg/mL, which was also observed in the cell metabolic assay (Figure 5). The samples that were in media with phosphate had absorbances that were two times as much in the higher zinc oxide concentrations than 10 µg/mL, which was observed in the cell metabolic assay as well.

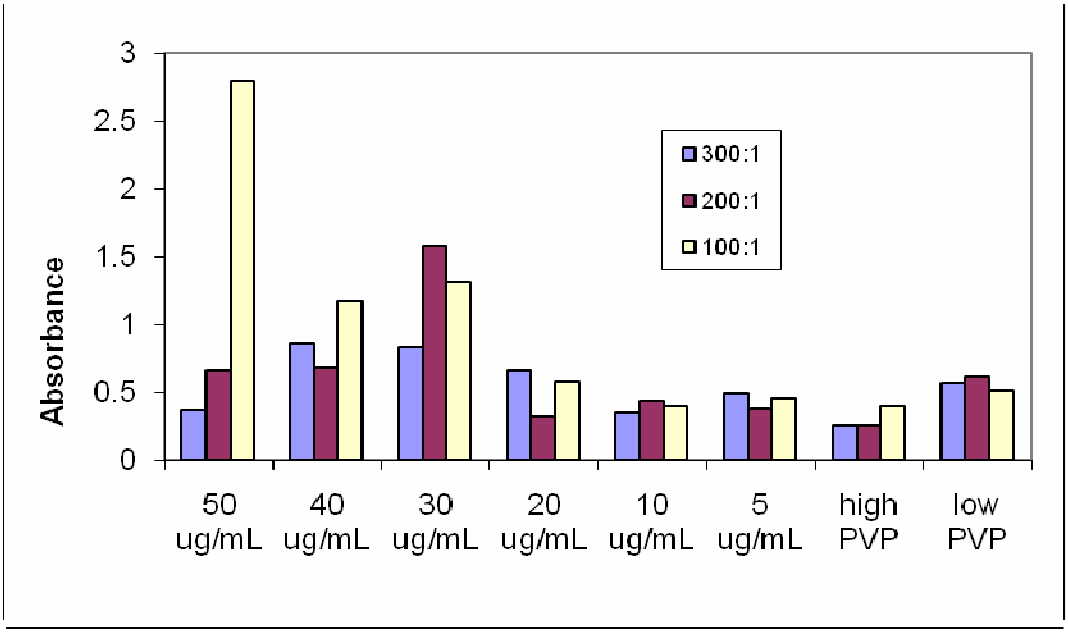


(a)

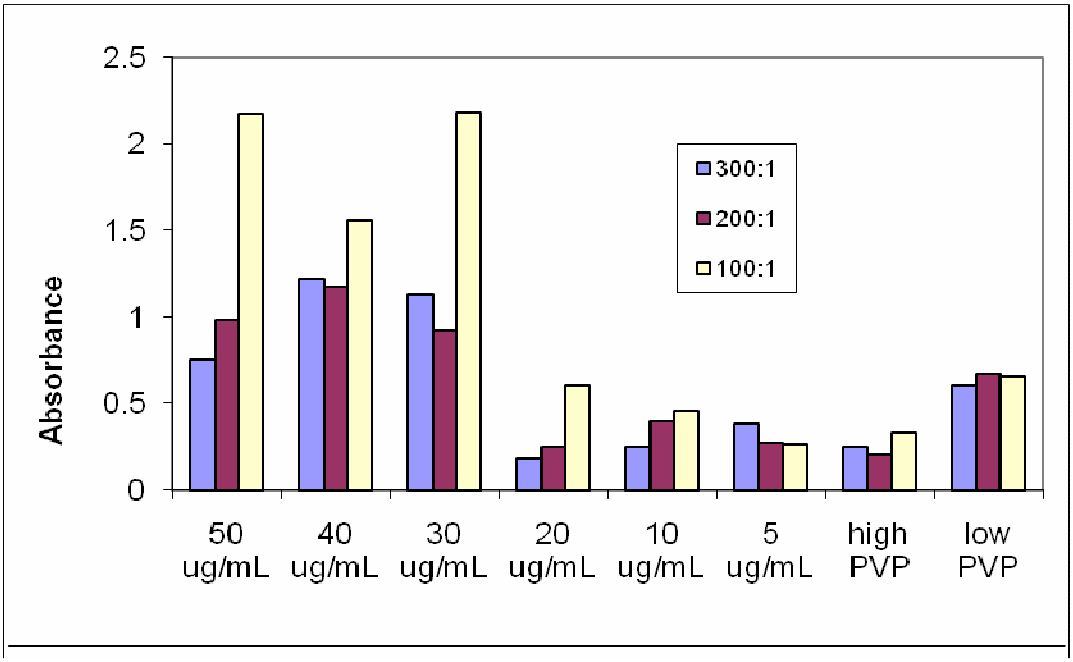


(b)

Figure 7. Viability/LDH Results of Cell Samples with: (a) DMEM with Phosphate. (b) DMEM without Phosphate.



(a)



(b)

Figure 8. Cell Death Detection ELISA Assay: (a) DMEM with Phosphate. (b) DMEM without Phosphate.

2.3 Efficiency of the Gene Delivery Vehicle

siRNA is a class of 20-25 nucleotide long double- stranded RNA molecules that are involved in the RNA interference (RNAi) pathway by interfering with the expression of a specific gene. The transfection of siRNA in clinical studies have not been successful mainly because of several reasons including the lack of an efficient targeted delivery, low transfection efficiency, instability to nucleases, poor tissue penetration, and nonspecific immune stimulation[25].

Plasmid DNA is a circular and double-stranded extra-chromosomal DNA molecule separate from the chromosomal DNA which is capable of replicating independently of the chromosomal DNA. They range from 1 to over 200 kilobase pairs in size and are favorable for gene transfer[26]. Plasmid DNA transfection has the same efficiency problems seen with siRNA, but their promising results make them a favorable method of treatment for certain illnesses.

The gene explored in this work is the serum response factor (SRF). SRF is a transcription factor that plays an important part in the development of the embryo. It has been found that SRF is linked to the formation of mesoderm, which is essential for embryonic development into a fetus[27]. For fully developed mammals, SRF has also been found to play a part in the growth of skeletal muscles[28]. It is seen for gene transfer of both plasmid DNA and siRNA that one of the major problems is the efficiency of the procedure. The genes have to escape from serum nucleases, endosomal entrapment, and pass through the nuclear membrane in order to reach the nucleus without damage for successful gene transfer[29]. The stability of the transferred gene in the nucleus is another

problem with gene transfer. There are viral and non-viral vectors for the delivery of genes in gene therapy. The benefit of non-viral vectors is that they are less immunogenic, can be easily produced, and have shown to have better stability. The downside of using non-viral vectors is that they are not as efficient as viral systems. In this work, we used a cationic lipid non-viral system Lipofectamine™ 2000 as the vector for the delivery of the SRF gene into the cells. Cationic lipids help with the delivery of the gene by forming a compact structure with its negatively charged phosphate backbone so it can enter the cell easily[30]. In addition to analyzing the samples for the presence of SRF, GAPDH will also be analyzed to allow comparison of SRF results. GAPDH is a protein involved in glycolysis and its presence in the extracted samples is usually used as an indicator of the quantity of viable cells[31].

2.3.1 Materials

C3H 10T 1/2 mouse mesenchymal stem cells[24] were subcultured at 37˚C/5% CO_2 in Dulbecco's modification of Eagle's medium (DMEM with 4.5 g/L glucose, L-glutamine and sodium pyruvate; Cellgro, Herndon, VA) supplemented with 10% heat inactivated fetal bovine serum (DFBS).

2.3.2 Methods

2.3.2.1 Maintenance of Cells

C3H 10T1/2 cells of passage 5-8 were used in the experiments. The cells were expanded and seeded by subculturing at 37˚C/5% CO_2 in DMEM supplemented with 10% DFBS, 1% PS. Twenty-four hours prior to transfection the cell culture media is

changed to DMEM supplemented only with 10% DFBS and no antibiotics. The cells were seeded at a density of 25,000 cells/cm^2 in a twenty-four well plate (B. D. Falcon, Franklin Lakes, NJ) for two-dimensional transfections.

2.3.2.2 Transfection

The transfection of SRF plasmid and siRNA were both explored in this study to see the effects of both extremes of upregulating and silencing a specific gene. Transfection procedure of the plasmid and siRNA were made based on the Lipofectamine™ 2000 reagent (Invitrogen, Carlsbad, CA) guidelines.

2.3.2.3 SRF Plasmid DNA

2.3.2.3.1 Two-Dimensional Environment

Each well had a final volume of 500 µL. Plasmid DNA was used at 150 ng/50 µL Ultraculture medium (Lonza, Walkersville, MD) in each well. 3 µL of Lipofectamine™ 2000 reagent was mixed with 150 µL of Ultraculture medium. The two solutions were then mixed and let sit at room temperature for twenty minutes. 100 µL of the mixture was added to each transfected well. The control wells received 500 µL of Ultraculture medium.

2.3.2.3.2 Three-Dimensional Environment

6,000 molecular weight poly (ethylene glycol) (PEG) gel was dissolved in HBS (10 mM HEPES + 150 mM NaCl, pH 7.4) at concentration of 10% (w/v) along with 3.9 mg/mL of cell adhesive peptide RGDS. 1% (w/v) of acetylphenone was added as a

photoinitiator. The PEG gel solution was then sterilized through filtration (0.22 μm, PVDF; Fisher Brand, Pittsburgh, PA). C3H 10T1/2 cells were trypsinized and resuspended in the sterilized PEG solution. 1 ml of cell suspension was rapidly pipetted between two sterile clamped glass plates separated by 0.75 mm spacers and exposed to UV light (365 nm, ~ 9 mW/cm^2) for 2 min for each side of the gel constructs. It has been shown that the longwave UV light used at similar intensities and exposure times for uniform photopolymerization results in minimal cell damage[32]. Roughly 7.5 million cells were encapsulated in each gel construct. The prepared hydrogel constructs were transferred to a culture plate and incubated at 37˚C/5% CO_2 in cell culture media (DMEM + 10% DFBS + 1% penicillin streptomycin amphotericin (PSA)). Change cell culture media to pure DMEM the day before transfection to diffuse out antibiotics in the gel.

20 μg of SRF plasmid DNA was dissolved in 500 μL of media and mixed with 50 μL Lipofectamine™ 2000 reagent that was diluted in 500 μL of media. The gels were washed with PBS (HyClone, Logan, UT) for ten minutes before adding the transfection solutions. The transfection solution was then added to 10 mL of media that the hydrogel was cultured in and incubated overnight. The control gel was treated the same with the exception of plasmid DNA in the solution. The media for the transfected samples was changed to DMEM with serum and antibiotics after six hours to prevent contamination.

Punch-out samples of the gels were collected after twenty-four hours, forty-eight hours, seventy-two hours, and 7 days of incubation with a biopsy punch (6 mm, Miltex,

York, PA). The samples were fast frozen in liquid nitrogen for about two minutes after collection and stored in -80 ^{o}C until RNA extraction.

2.3.2.4 SRF siRNA

2.3.2.4.1 Two-Dimensional Environment

The final volume was 500 μL in each well. 20 pmol of SRF siRNA + 1 μL of Lipofectamine^{TM} 2000 reagent were added to each well. 2 μL of SRF siRNA was diluted in 50 μL of transfection medium (Santa Cruz Biotechnology, Santa Cruz, CA) and mixed gently. 1 μL of Lipofectamine^{TM} 2000 reagent was diluted in 50 μL of transfection medium, mixed gently, and incubated at room temperature for five minutes. The two solutions were combined, mixed gently, and incubated at room temperature for twenty minutes. The cells in the wells were washed once with 2 mL of PBS. 100 μL of the transfection mixture was mixed with 400 μL of transfection medium and placed in wells for transfection. Control wells were treated the same with the exception of no siRNA in the solution. The cells were incubated for five-seven hours and media was changed to DMEM + 10% DFBS + 1% PSA. The cells were checked with a confocal microscope for fluorescence after twenty-four hours of incubation to see if transfection was successful because the control siRNA contains a fluorescent tag. Pictures of the cells were taken.

2.3.2.4.2 Three-Dimensional Environment

6,000 molecular weight PEG gel was dissolved in HBS at concentration of 10% (w/v) along with 3.9 mg/mL of cell adhesive peptide RGDS. 1% (w/v) of acetylphenone

was added as a photoinitiator. The PEG gel solution was then sterilized through filtration. C3H 10T1/2 cells were trypsinized and resuspended in the sterilized PEG solution. The hydrogels were made in sterile forty-eight well plates by exposure to UV light for five minutes. The gels were made in wells instead of between plates to ensure a RNAse free environment. The hydrogels were then washed with PBS for five minutes and then PBS + 1% PSA to prevent possibility of contamination. The hydrogels were transferred to a twenty-four well plate with DMEM + 10% DFBS + 1% PS and incubated overnight. The media was changed to one without antibiotics the day prior to transfection. The same procedure was carried out as in the two – dimensional transfection for siRNA. The cells were checked with a confocal microscope for fluorescence after twenty-four hours of incubation to see if transfection was successful and pictures were taken for all samples. The hydrogel samples were collected by cutting each gel in half and fast frozen in liquid nitrogen for about two minutes and stored in -80 ^{o}C for RNA extraction at a later date.

2.3.2.5 RNA Extraction

2.3.2.5.1 Two-Dimensional Environment

RNeasy® Mini Kit (Qiagen Sciences, Valencia, CA) was used for the extraction of RNA from the samples. 70% RNA free ethanol was prepared before the experiment. The hood and all equipment are wiped with RNase away to avoid contamination and degradation of the RNA in the samples. The cell culture media was removed and the wells were washed with PBS. 1mL/10cm^2 Trizol (Invitrogen, Carlsbad, CA) was added

to each well and the wells were incubated for five minutes at room temperature. The Trizol extract was transferred to a new tube and 0.2mL chloroform/mL Trizol was added to the tubes, shook vigorously for fifteen seconds and incubated at room temperature for three minutes. The samples were centrifuged at 11,000 rpm for fifteen minutes at 4 ^{o}C. The supernatant was then transferred to a new tube and 1:1 volume of 70% ethanol was added and mixed. The sample was then transferred to an RNeasy column and centrifuged at 11,000 rpm at room temperature for fifteen seconds. 700 µL of Buffer RW1 was added to the RNeasy column and the column was centrifuged at 11,000 rpm for fifteen seconds to wash the column. The column was then transferred to a new collection tube and 500 µL Buffer RPE was added to the RNeasy column. The column was centrifuged for fifteen seconds at 11,000 rpm. 500 µL Buffer RPE was added to the column again, but was centrifuged for two minutes instead. The column was then placed in a new collection tube and centrifuged at full speed for one minute. The column was then transferred to a new collection tube and 30 µL of RNase-free water was placed in the column to elute the RNA. The column was centrifuged at 11,000 rpm for fifteen seconds and the 30 µL collected was put through the column again to elute the RNA. The collected eluted RNA was split into 25 µL and 5 µL quantities and stored in -80 ^{o}C for later analysis.

2.3.2.5.2 Three-Dimensional Environment

500 µL of Trizol reagent was placed into a tube with the samples. Approximately 1 mL of stainless steel beads were added to the tubes and another 1 mL of Trizol was added. The samples were then homogenized by using a micro beater at 48,000 rpm for

10 seconds while keeping the samples on ice for at least thirty seconds afterwards to prevent the heating of the samples. This cycle was repeated nine times until the gels were mashed into small pieces. The solution was then removed from the tubes and transferred to a new tube. The tube was centrifuged at 10,000 rpm for ten minutes at 4 ^{o}C. The supernatant was transferred into a 2 mL RNase free conical tube and the samples were incubated for five minutes at room temperature. 300 µL of chloroform was added and the tubes were shook vigorously for fifteen seconds and incubated for three minutes at room temperature. The tubes were then centrifuged at 10,000 rpm for fifteen minutes at 4 ^{o}C. The supernatant was transferred into new conical tubes and add 1 µL of 4 µg/µL glycogen for each 10 µL RNA sample. 10 µL was used due to the large volume. 750 µL of isopropanol was added and mixed. The sample was incubated at -20 ^{o}C for an hour and a half.

2.3.2.5.2.1 Cleaning RNA

Centrifuge samples at 10,000 rpm at 4 ^{o}C for twenty minutes. The supernatant was discarded without disturbing the pellet and 1.5 mL of 75% RNase free ethanol was added. The samples were centrifuged at 8,000 rpm at 4 ^{o}C for ten minutes. The supernatant was discarded without disturbing the pellet and 1.5 mL of 95% RNase free ethanol was added. The samples were centrifuged at 8,000 rpm at 4 ^{o}C for ten minutes. The supernatant was discarded without disturbing the pellet and let dry for ten minutes at room temperature.

2.3.2.5.2.2 DNase Treatment

The pellet was resuspended in 87.5 µL of RNase free water, 10 µL of RDD buffer, and 2.5 µL of DNase I. The samples were incubated at 37 ^{o}C for thirty minutes and then transferred to a 70 ^{o}C oven for five minutes to inactive the enzyme and immediately chilled on ice. 10 µL of 3M NaAc at pH 5.5 was added. 275 µL of 100% RNA free ethanol was then added and incubated overnight at -20 ^{o}C.

2.3.2.5.2.3 Second Precipitation

The samples were centrifuged at 10,000 rpm for twenty-five minutes at 4 ^{o}C. The supernatant was removed without disturbing the pellet. 500 µL of 75% RNase free ethanol was added and centrifuged at 8,000 rpm for ten minutes at 4 ^{o}C. The supernatant was discarded without disturbing the pellet. 500 µL of 95% RNase free ethanol was added and centrifuged at 8,000 rpm for ten minutes at 4 ^{o}C. The supernatant was discarded without disturbing the pellet and let dry for ten minutes at room temperature. The pellet was dissolved in 30 µL of RNase free water and stored in -80 ^{o}C if not running RT-PCR immediately.

2.3.2.5.2.4 Protein Dialysis

450 µL of ethanol was added to each tube containing protein stored from earlier. Each tube was mixed gently by inversion. The DNA pellet was collected by centrifuging at 2,000 Xg for ten minutes at 4 ^{o}C. The samples were dialyzed with 0.1% SDS buffer at 4 ^{o}C over a period of two days with a change of buffer after one day.

Snake Skin® Pleated Dialysis Tubing (3,500 MW, Thermo Fisher Scientific, Rockford, IL) was used as the membrane for the dialysis.

2.3.2.6 RNA Quantification

Quant-iT™ RNA Assay Kit (Invitrogen, Eugene, OR) was used to quantify the RNA concentration extracted from the samples. The working solution was diluted 1:200 and 200 μL was added to each well for each sample. 10 μL of each standard (0 ng and 100 ng) were added to the wells with working solution and 5 μL of the RNA samples were added to the wells with working solution. The fluorescence at 644/673 nm were read at a sensitivity of twelve with a Spectra Max Gemini EM Spectrofluorometer (Molecular Devices, Union City, CA).

2.3.2.7 RT-PCR

SuperScript™ III Platinum® SYBR® Green One-Step qPCR System (Invitrogen, Eugene, OR) was used to carry out the RT-PCR in order to compare and quantify the RNA in the samples. Duplicates were made for each sample because both GAPDH (human glyceraldehydes-3-phosphate dehydrogenase) and SRF genes are amplified. Samples were prepared and loaded into the Applied Biosystems StepOne™ Real-Time PCR machine (Applied Biosystems, Foster City, CA). A comparative C_T was made for the RT-PCR samples. This quantitation method determines the relative target quantity in samples. The SYBR® Green 1 dye used is a fluorescent-based reagent that generates a fluorescence signal when bound to double-stranded DNA, which will then allow the quantification of PCR products through the cycles[33].

2.3.2.8 Protein Quantification

Coomassie (Bradford) Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL) was used to quantify the amount of protein in the samples. 5 µL of each standard and sample were added to 250 µL of Coomassie Reagent. The absorbance was measured at 595 nm with a Multi-Detection Microplate Reader.

2.3.2.9 Western Blot

10% of 6 mL of the resolving gel for Tris-glycine SDS-polyacrylamide and 3 mL of 5% of the stacking gel were used for gel electrophoresis. The samples were prepared by adding 5X sample buffer and diluting it to 1X with the samples, and boiled in water for ten minutes. 5 µL of stain marker was loaded on the side. SDS-PAGE electrophoresis running buffer was used and the gel was ran at 100 volts for twenty minutes and then 180 volts for one hour. The gel was removed from the cassette and the samples were transferred onto a membrane for Western Blotting. The pads, filter paper, and membrane were soaked in the transfer buffer before assembling. 1 X Novex® Tris-Glycine transfer buffer was used. The materials were stacked with two blotting pads on the bottom, a filter paper, gel, transfer membrane, filter paper, and three blotting pads on top. The transfer was run at twenty-five volts for an hour and a half. The membrane was then washed with double DI water for five minutes twice on a rotary shaker. The membrane was soaked in blocking solution 3% BSA for sixty minutes on a rotary shaker. The membrane was rinsed with TBST for ten minutes. The membrane was cut in half between 55and 43 kDa according to the ladder (GAPHD is around 36 kDa and SRF is around 51 kDa[31]) and was incubated overnight in primary antibody

solution (1:200 SRF and 1:200 GAPDH, Santa Cruz Biotechnology, Santa Cruz, CA) at 4 ^{o}C. The top half of the membrane was incubated with SRF primary antibody and the bottom half of the membrane was incubated with GAPDH primary antibody.

The membrane was washed for ten minutes with TBST and repeated twice. The membrane was then incubated at room temperature with the secondary antibody solutions (1:10,000 anti-mouse HRP for GAPDH and 1:10,000 anti-rabbit HRP for SRF, Santa Cruz Biotechnology, Santa Cruz, CA) for sixty minutes. The membrane was washed with TBST for ten minutes and repeated twice. The membrane was washed with TBS for ten minutes and repeated once. The membrane was then placed on a sheet of transparent plastic and a mixture of Western Blotting Luminol Reagent (Santa Cruz Biotechnology, Santa Cruz, CA) was applied to the surface. The reaction was allowed to develop for a minute and another sheet of transparent plastic was placed on top. The membrane was exposed to the film for four minutes in the dark room. The film was immersed in the developer solution for about one minute and washed in double DI water for about one minute and soaked in the fixer solution for two minutes with shaking. The film was then washed with double DI water and air dried.

2.3.2.10 Gel Electrophoresis of RT-PCR Samples

35 mL of 2% agarose gel was prepared for the gel electrophoresis. The agarose powder was dissolved in 35 mL of 1X TAE buffer in a beaker, which was covered with a paper towel and secured with a rubber band. The beaker was placed in a microwave and heated for ten seconds. This was repeated three times with shaking and mixing of the beaker after each time. The mixture was let cool at room temperature and then

poured into the mold with an appropriate comb. After thirty minutes the mold was placed in an electrophoresis cartridge and filled with buffer solution. The wells were loaded with 5 μL of markers and 12μL of each sample (2 μL loading buffer + 10 μL sample). The gel was run at 100 volts for approximately forty-two minutes. The gel was then placed in ethidium bromide solution for forty minutes with rocking. The gel was washed with buffer for ten minutes with rocking before imaging.

The gel was then imaged with the Versa Doc imager machine (Bio-Rad, Hercules, CA) and a photo was taken.

2.3.3 Results

2.3.3.1 Two-Dimensional Environment

The cells were observed twenty-four hours after transfection and were found to fluoresce under the microscope with siRNA, which suggested the transfection was successful (Figures 9 and 10).

The mRNA extracted and purified from the SRF plasmid DNA transfection experiment was quantified and the results are presented in Table 2 in the Appendix. This shows an increase in amount of mRNA produced as time is increased, which suggests an increase in the number of cells and viability. The proteins collected from the SRF plasmid DNA transfection samples were analyzed with a Western Blot to see if the transfection was successful by comparing the expressions and the picture is shown as Figure 11.

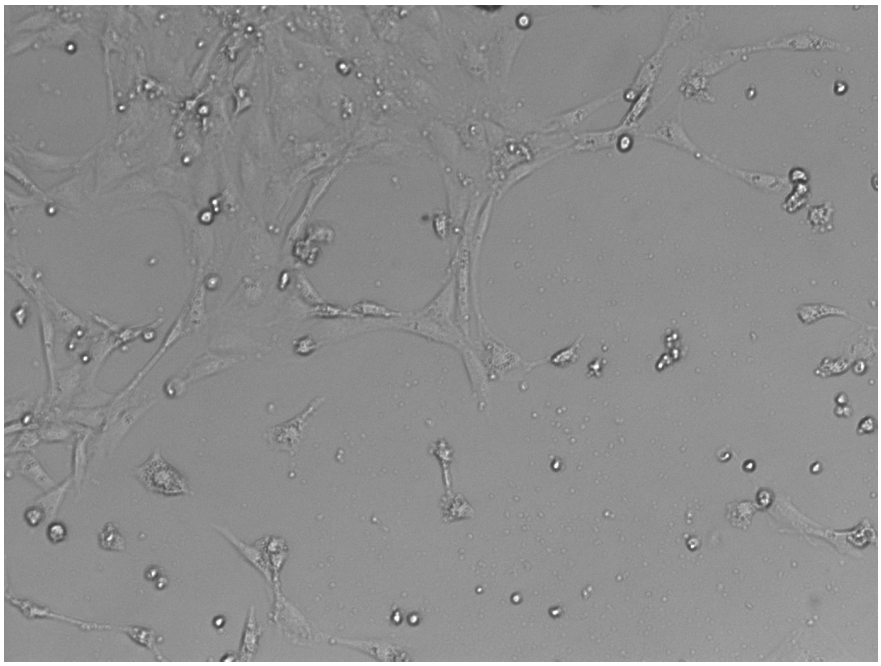


Figure 9. C3H 10T1/2 Cells 24 Hours after Transfection (Brightfield).

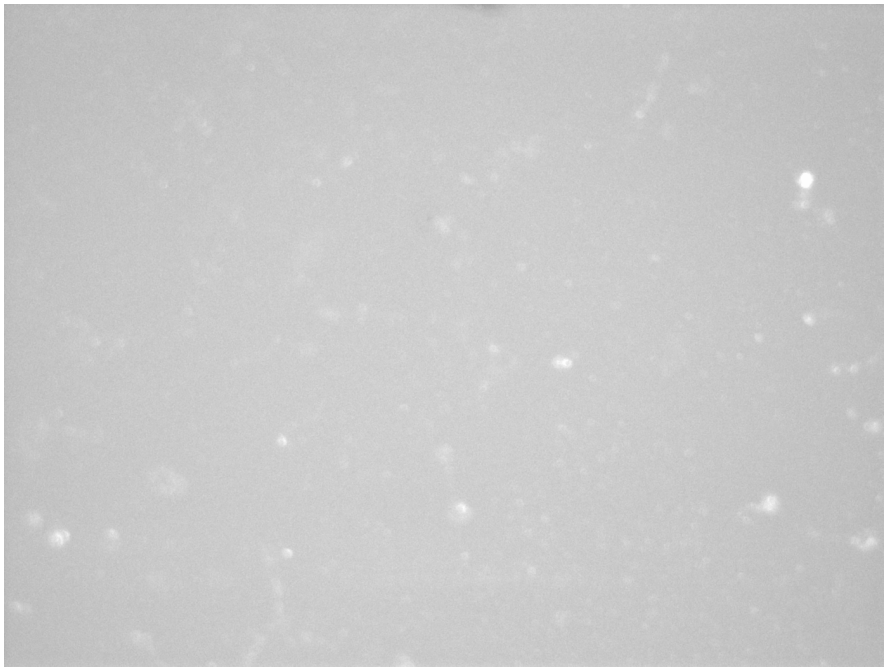


Figure 10. C3H 10T1/2 Cells 24 Hours after Transfection (FITC).

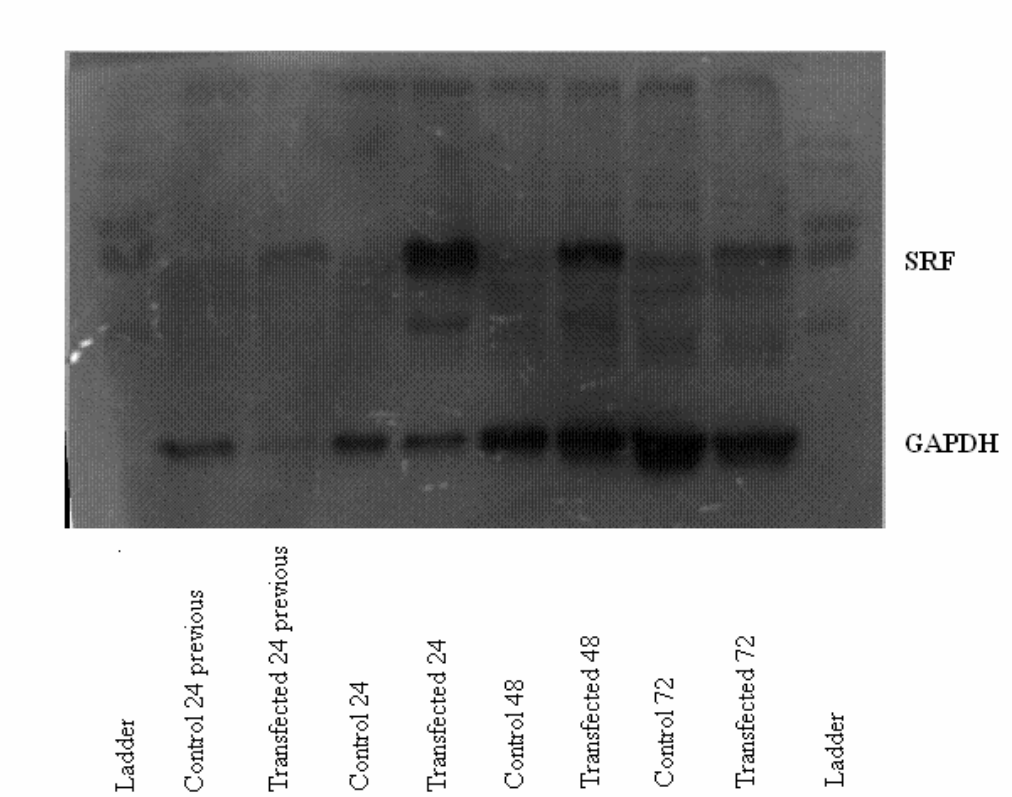


Figure 11. Photo of Western Blot of Protein Phases from 2-D Transfection.

It can be seen from the Western Blot that there is an amplified expression of GAPDH with the increase of time, which suggests an increase in cell number and viability that is seen as the amount of time the cells are incubated is increased. The expression of SRF is generally higher in the transfected sample than in the control, which suggests that the transfection was successful. The control SRF expression is also observed to increase with time, which also suggests an increase in cell number and viability as length of time increases. It is also observed that the amplified expression of SRF in transfected samples decreased through time, which is expected as the inserted gene is not very stable and will be expelled from the cell through time.

The mRNA extracted and purified from the SRF siRNA transfection experiment was quantified and the results are presented in Tables 3 and 4 in the Appendix. It was observed that the control wells appear to have been contaminated, which resulted in the very low mRNA quantification reading for the forty-eight hour control sample. The other samples seem to have a good amount of RNA present with an increase in the amount of RNA for the forty-eight hour sample, which confirms an increase in the amount of cells with time.

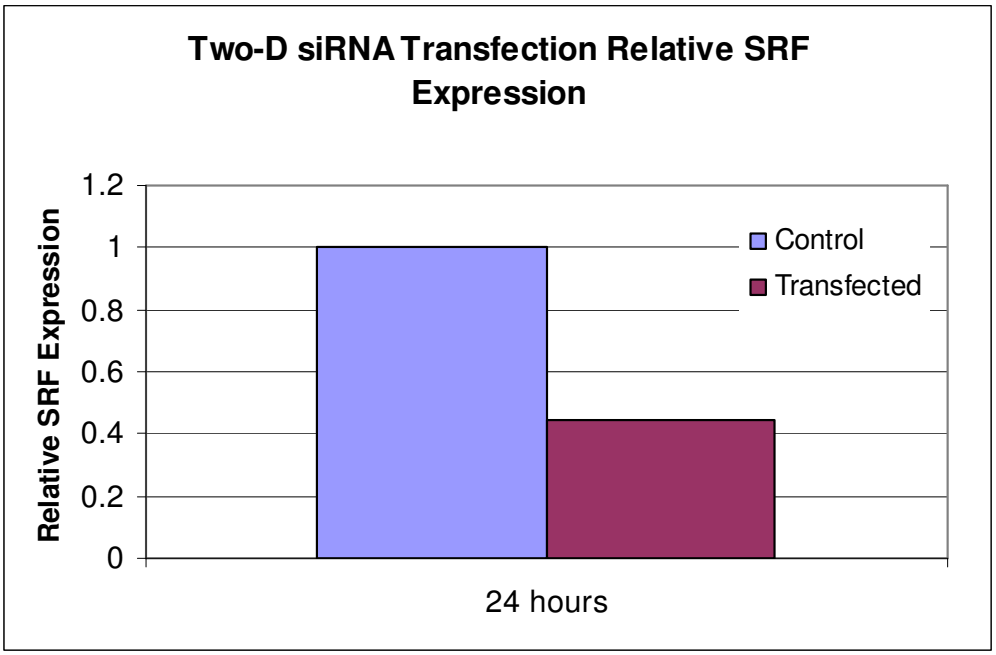


Figure 12. 2-D siRNA Transfection Relative SRF Expression after 24 Hours.

Initial analysis of the C_T from RT-PCR results from the siRNA transfection after twenty-four hours is presented in Figure 12. There is a 55% decrease in SRF expression in the transfected cells, but a decrease around 70% was expected for the time

period[34], which means fine tuning of the transfection procedure is needed.Due to a contamination in the control sample for the transfection and limited time to repeat the experiment, the data collected from the RT-PCR results could not be used to analyze the effects of forty-eight hours after transfection. An electrophoresis agarose gel of the RT-PCR samples for twenty-four hours after transfection showed clean bands for SRF and GAPDH in the samples (Figure 13), which confirmed the products from the RT-PCR. However, an electrophoresis agarose gel of the RT-PCR samples for forty-eight hours after transfection did not show clean distinct bands of SRF and GAPDH in the samples as seen in the previous gel (Figure 14), which was reflected in the RT-PCR results and is due to the contamination in the control sample.

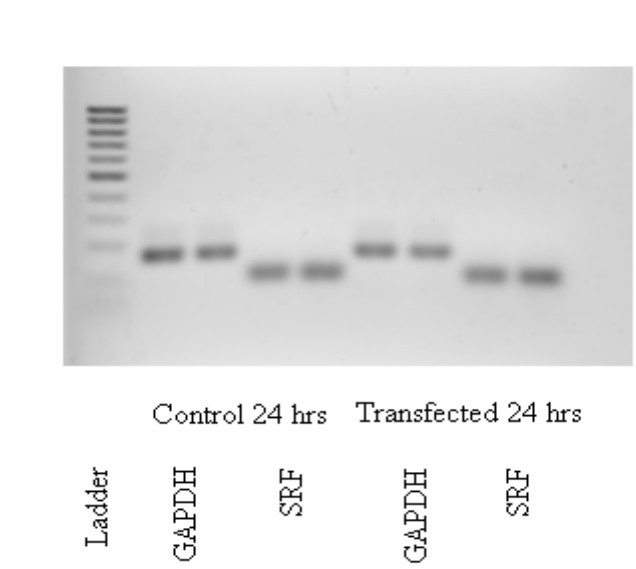


Figure 13. Gel Electrophoresis of RT-PCR Results for 24 Hrs after Transfection.

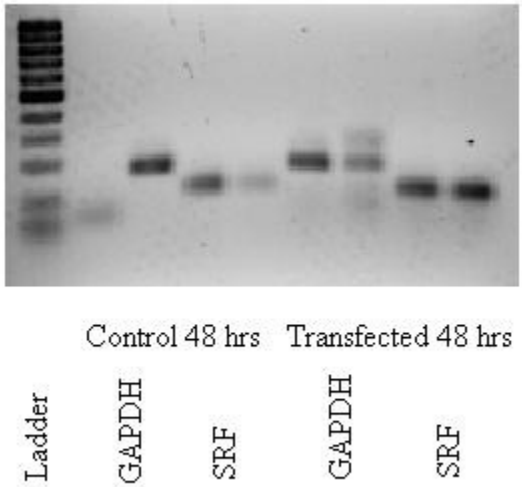


Figure 14. Gel Electrophoresis of RT-PCR Results for 48 Hrs after Transfection.

2.3.3.2 Three-Dimensional Environment

The cells were observed through the microscope twenty-four hours after transfection and were found to show no fluorescence for the siRNA, which suggested the transfection was not successful. However, analysis of the extracted mRNA showed that transfection was successful.

The mRNA extracted and purified from the SRF plasmid DNA transfection experiment was quantified and the results are presented in Table 5 in the Appendix. RT-PCR results were promising even though the amount of SRF expression was observed to be down 4.4% compared to the control after twenty-four hours but was up 12.11% after 168 hours, which suggest the transfection was fairly successful since an increase in SRF expression was still observed after seven days (Figure 15). Only the samples from twenty-four hours and seven days were analyzed in order to analyze both the short-term and long-term effects of the transfection.

The mRNA extracted and purified from the SRF siRNA transfection experiment was quantified and the results are presented in Table 6 in the Appendix. RT-PCR results also did not yield very good results where no data was obtained from the control samples and only three amplifications values were obtained for the transfected samples for after twenty-four hours of transfection, which cannot be used for analysis due to lack of data. The RT-PCR transfection data from forty-eight hours show that there is a 1.3% reduction in the expression of SRF compared to the control (Figure 16). This means that either the siRNA was successfully transfected in the cells but were already degraded by forty-eight hours or that the transfection was not successful at all.

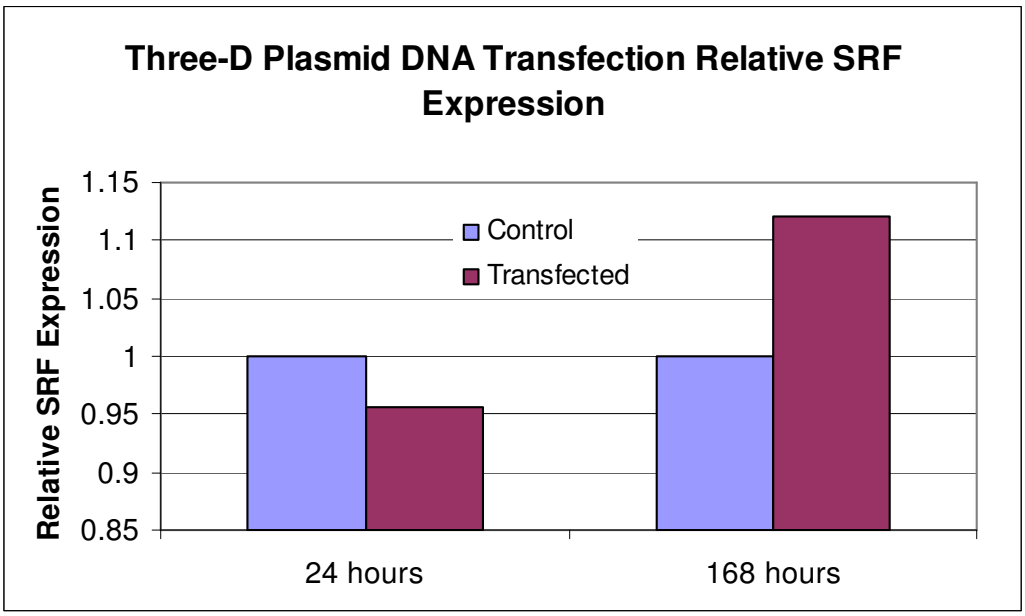


Figure 15. 3-D Plasmid DNA Transfection Relative SRF Expression after 24 and 168 Hours.

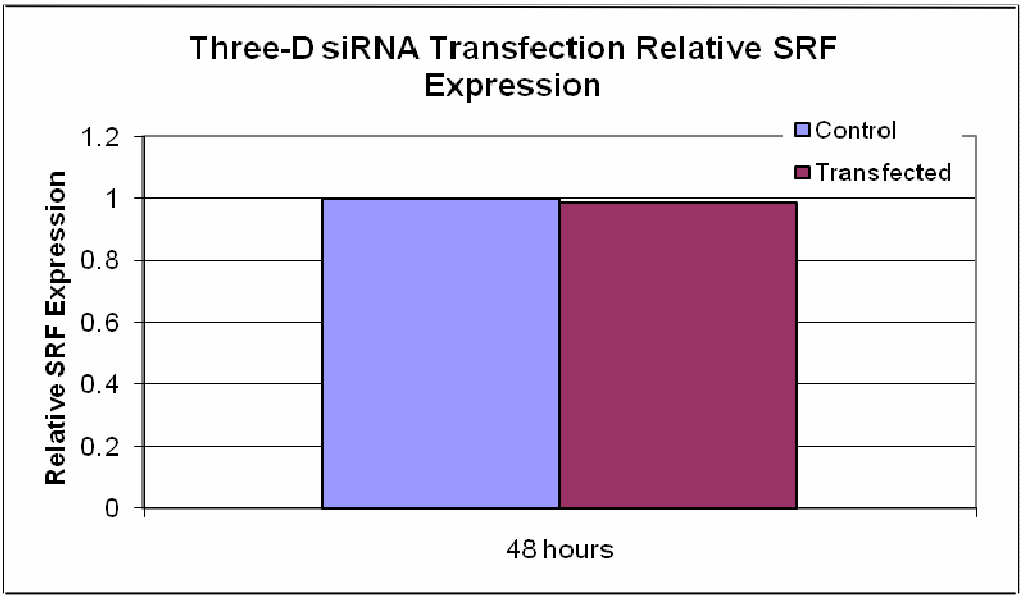


Figure 16. 3-D siRNA Transfection Relative SRF Expression after 48 Hours.

It was seen that there is a minimal amount of RNA extracted compared to the standards and the Western blots showed little or no bands. The experiment was repeated with a second set of treatments where the transfection media was not changed after six hours of incubation in order to observe the effects of a longer transfection period.

The results from the Western blot from the three-dimensional transfection show that there seems to be a higher concentration of SRF present in the sample that media was not changed after transfection (Figure 17). GAPDH bands were not visible in the Western blot and different antibodies will be used in the future. Due to the lack of GAPDH data we could not normalize the SRF expression and normalized the data with each other with the Scion Image program. After normalizing the data and analyzing the bands it was found that there is a significant decrease in SRF expression between the transfected sample and the control where the media was not changed after twenty-four hours (Figure 18)[35]. It was also seen that there was little difference between the control and transfected in the forty-eight hour sample, which could be due to the decrease in cell viability for being in the gel and the increase in SRF production by the cells through time after siRNA is degraded. This information tells us that the effective time frame for this transfection method would be between twenty-four and forty-eight hours.

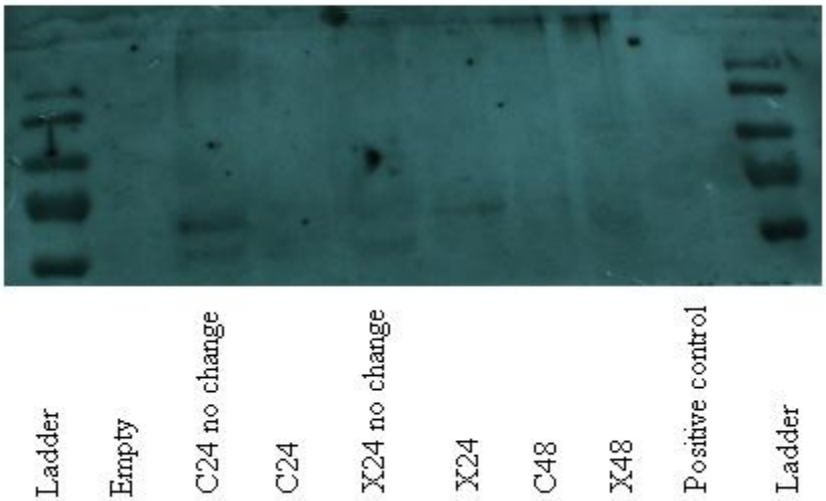


Figure 17. Photo of Western Blot of Protein Phases from 3-D Transfection (SRF Half).

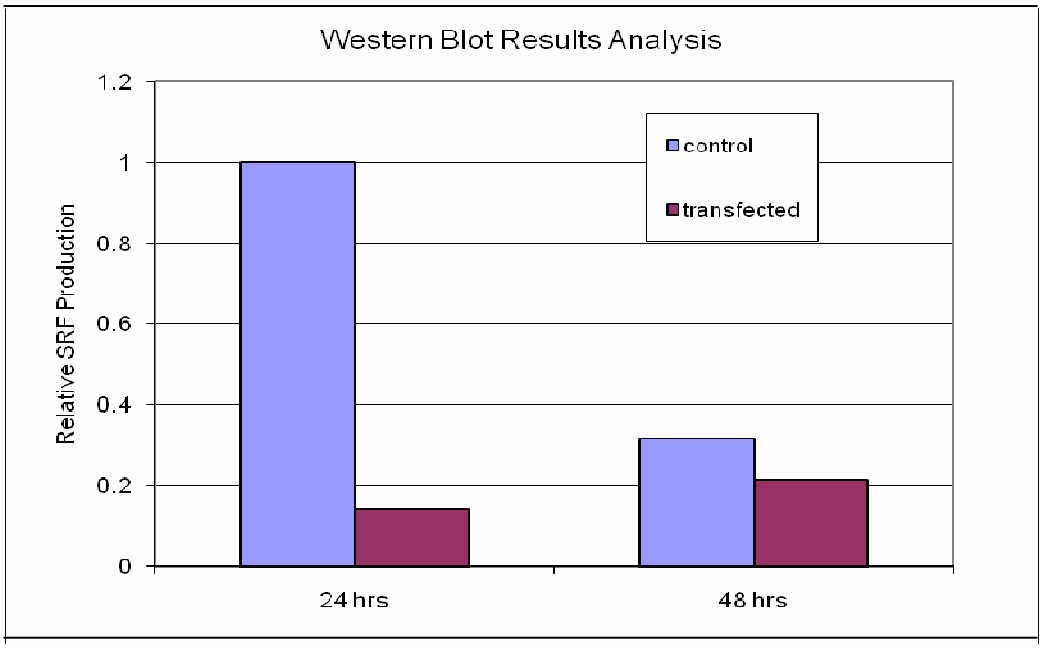


Figure 18. Western Blot Quantification Results from 3-D Transfection.

3. DISCUSSION

Novel and efficient drug delivery methods are currently being explored as alternative ways to administer treatment for a wide range of patients with different conditions. Certain delivery methods are more favorable than others due to the nature of the drug and the location of its target site. Aspects of a controlled release gene delivery system from an implantable device were explored in this work. It was seen that the degradation times for the same polymer concentration vary from time to time and is not consistent. This is one of the challenges when finding a suitable polymer material for a timed-release drug delivery module. In this work, we hypothesized that 50/50 DLG 5A would have the longest degradation time, but it was observed to have degraded within the first few days of several experiments. The next polymers to have a longer degradation time should be 50/50 DLG 4A, 50/50 DLG 2.5A, 65/35 DL 3A, 75/25 DLG 2A, 85/15 DLG 1A, and finally 75/25 DLG 1A according to their properties. However, this was not observed in the several experiments we carried out for all seven polymer compositions made the same way at the same time. Instead, 65/35 DL 3A and 50/50 DLG 4A were the two compositions that had a degradation time of around two weeks. This could be largely due to the difficulty of maintaining a consistent thickness in the polymer membrane created. It was also observed by Grayson et al[12] that certain polymer compositions did not exhibit expected behavior during her work.

The results presented here show that for zinc oxide nanoparticles with a PVP coated layer the cytotoxicity increases as the concentration of zinc oxide increases and that the presence of PVP may play a role in the cytotoxic effects. The significant

decrease in cell viability between 10 and 20 μg/mL could be due to the covering of cells by the nanoparticles, thus, impeding the ability for nutrients to reach the cells on the bottom of the wells, which was also observed in previous studies[36]. It was also shown that the method of cell death is very likely through apoptosis since it is a programmed cell death that results in the fragmentation of DNA[37], which was observed that a high level of low cell viability is associated with a high level of DNA fragmentation. It is unlikely for apoptosis to take place if the cell walls were punctured and ruptured since apoptosis takes time to carry out since it is a programmed cell death. These findings do not support the theory that an increase in PVP causes an increase in the number of cell death through the interactions between the zinc oxide particles and phosphate moleculeus to create zinc phosphate crystals that puncture cell walls.

Also presented in this work is the feasibility of the gene transfection of SRF plasmid DNA and siRNA with a lipid-based non-viral vector. It was found that the transfection of the plasmid DNA and siRNA was semi-successful in the three-dimensional environment and fairly successful in the two-dimensional environment. This could be due to the transfection method used in the experiments where a lipid vector was used, which reduced the transfection efficiency[30]. In order for the gene to be transfected into the cell in a three-dimensional environment the liposome encapsulated gene has to be able to reach the cells to fuse with the cell wall and transport the gene into the nucleus (Figure 2), but because the cells were encapsulated in a PEG gel construct it was difficult for the liposomes to be able to come in contact with most of the cells, thus, reducing the efficiency of the transfection. However, it was found that the transfection

of the siRNA of SRF was fairly successful in the three-dimensional environment within a twenty-four to forty-eight hour time frame, and this information allows us to see that the Lipofectamine™ 2000 transfection method is feasible with the siRNA in the three-dimensional environment within the time frame.

4. CONCLUSION AND FUTURE RESEARCH

The aim of this study was to explore three key aspects of gene therapy for its application in tissue engineering. It is important to be able to control the release of the drugs within the body so that optimal therapeutic levels can be obtained. It is also important to assess the cytotoxicity of any materials used in conjunction with the delivered gene to understand the interaction of the materials and the surrounding tissues and organs to prevent adverse side effects. Lastly, it is important to investigate the efficacy of the delivery system designed to assess the feasibility of taking the method to the next level.

Designing a biodegradable timed-release microchip with multiple wells for loading drugs is ideal for certain applications, but finding an appropriate polymer composition to achieve a certain degradation time and maintain a constant membrane thickness for the situation is difficult. 12% 50/50 DLG 4A and 65/35 DL 3A can be tested with drugs to observe the release rates. Other formulations than the ones tested could also be explored.

The cytotoxicity of the zinc oxide nanoparticles is significant at higher concentrations of the nanoparticles and lower concentrations of PVP, which gives us valuable information regarding the threshold concentration of zinc oxide that should be used when using them as an aid in delivering the genes to their target sites.

The concept of gene therapy is in its initial stages and has not yet been proven to be an effective method of treating a variety of illnesses and diseases. It was seen in this work that it is difficult to transfect the plasmid DNA or siRNA in a three-dimensional

environment especially when the inserted gene is usually rejected within a short time period (within twenty-four hours). The effects of the inserted gene would be short and it was observed to be somewhere between twenty-four and forty-eight hours as the optimal therapeutic time frame. Alternative methods of transfection that could be considered would be to use viral vectors instead of non-viral vectors to increase efficiency.

These findings and results from the above proposed research will benefit the continual search for a better gene therapy drug delivery method as well as currently explored methods.

REFERENCES

1. Saltzman, W. M. *Drug Delivery: Engineering Principles for Drug Therapy*; Oxford University Press: New York, 2001.

2. Naik, A.; Kalia, Y. N.; Guy, R. H. *PSTT* **2000**, *3*, 318-326.

3. Azarmi, S.; Roa, W. H.; Lobenberg, R. *Advanced Drug Delivery Reviews* **2008**, *60*, 863-875.

4. Genetics Home Reference. What Is Gene Therapy? http://ghr.nlm.nih.gov/handbook/therapy/genetherapy (accessed on August 14, 2008).

5. Mukai, H.; Kawakami, S.; Hashida, M. *Biochemical and Biophysical Research Commnunications* **2008**, *372*, 383-387.

6. Vaxim. Gene Therapy/DNA Delivery. http://www.vaxim.com/gene_therapy_DNA_del.htm (accessed on August 14, 2008).

7. Grayson, A. C. R.; Choi, I. S.; Tyler, B. M.; Wang, P. P.; Brem, H.; Cima, M. J.; Langer, R. *Nature* **2003**, *2*, 767-772.

8. Slowing, I. I.; Vivero-Escoto, J. L.; Wu, C.-W.; Lin, V. S.-Y. *Advanced Drug Delivery Reviews* **2008**, *60*, 1278-1288.

9. Efentakis, M.; Koligliati, S.; Vlachou, M. *International Journal of Pharmaceutics* **2006**, *311*, 147-156.

10. Wang, X.; Zhang, X.; Castellot, J.; Herman, I.; Iafrati, M.; Kaplan, D. L. *Biomaterials* **2008**, *29*, 894-903.

11. Liu, X.; Pettway, G. J.; McCauley, L. K.; Ma, P. X. *Biomaterials* **2007**, *28*, 4124-4131.

12. Grayson, A. C. R. PhD. Dissertation, Massachusetts Institute of Technology, 2003.

13. Stevens, M. P. *Polymer Chemistry: An Introduction*; Oxford University Press: New York, 1999.

14. Gilding, D. K.; Reed, A. M. *Polymer* **1979**, *20*, 1459-1464.

15. Royte, E. *Smithsonian Magazine* **2006**, *37*, 84-88.

16. Lakeshore Biomaterials. Products: How Polymers Degrade. http://www.lakeshorebiomaterials.com/ (accessed on August 27, 2008).

17. Warheit, D. B.; Sayes, C. M.; Reed, K. L.; Swain, K. A. *Pharmacology and Therapeutics* **2008**, *120*, 35-42.

18. Hu, T.-T.; Wang, J.-X.; Shen, Z.-G.; Chen, J.-F. *Particuology* **2008**, *6*, 239-251.

19. Hawkins, M. J.; Soon-Shiong, P.; Desai, N. *Advanced Drug Delivery Reviews* **2008**, *60*, 876-885.

20. Chertok, B.; Moffat, B. A.; David, A. E.; Yu, F.; Bergemann, C.; Ross, B. D.; Yang, V. C. *Biomaterials* **2008**, *29*, 487-496.

21. Bai, J.; Li, Y.; Zhang, C.; Liang, X.; Yang, Q. *Colloids and Surfaces A: Physicochemical and Engineering Aspects* **2008**, *318*, 275-284.

22. Gulson, B.; Wong, H.; McCall, M.; Casey, P.; Trotter, J.; McCulloh, M.; Greenoak, G.; Stauber, J. *Toxicology Letters* **2008**, *180*, S222.

23. Thermo Fisher Scientific, Inc. HyClone Media: Penicillin/Streptomycin.

http://www.hyclone.com/media/pen-strep.htm (accessed on August 21, 2008).

24. ATCC. ATCC: A Global Bioresource Center. http://www.atcc.org/ATCCAdvancedCatalogSearch/ProductDetails/tabid/452/Default.aspx?ATCCNum=CCL-226&Template=cellBiology (accessed on August 21, 2008).

25. Pirollo, K. F.; Chang, E. H. *Cancer Research* **2008**, *5*, 1247-1250.

26. Lipps, G. *Plasmids: Current Research and Future Trends*; Caister Academic Press: United Kingdom, 2008.

27. Barron, M. R.; Belaguli, N. S.; Zhang, S. X.; Trinh, M.; Iyer, D.; Merlo, X.; Lough, J. W.; Parmacek, M. S.; Bruneau, B. G.; Schwartz, R. J. *Journal of Biological Chemistry* **2005**, *12*, 11816-11828.

28. Li, S.; Czubryt, M. P.; McAnally, J.; Bassel-Duby, R.; Richardson, J. A.; Wiebel, F. F.; Nordheim, A.; Olson, E. N. *Proceedings of the National Academy of Sciences of the United States of America* **2005**, *4*, 1082-1087.

29. Canine, B. F.; Wang, Y.; Hatefi, A. *Journal of Controlled Release* **2008**, *129*, 117-123.

30. Dal-Maso, A. D.; Dellacasagrande, J.; Legendre, F.; Tiraby, G.; Blonski, C.; Hoffmann, P. *European Journal of Medicinal Chemistry* **2008**, *43*, 1758-1766.

31. Cell Signaling Technology. *PhosphoSite Plus*. http://www.phosphosite.org/proteinAction.do?id=1548&showAllSites=true (accessed on August 27, 2008).

32. Hahn, M. S.; Taite, L. J.; Moon, J. J.; Rowland, M. C.; Ruffino, K. A.; West, J. L. *Biomaterials* **2006**, *27*, 2519-2524.

33. Applied Biosystems. *Applied Biosystems StepOne™ and StepOnePlus™ Real-Time PCR Systems: Relative Standard Curve and Comparative C_T Experiments*; Applied Biosystems: Foster City, CA, 2007.

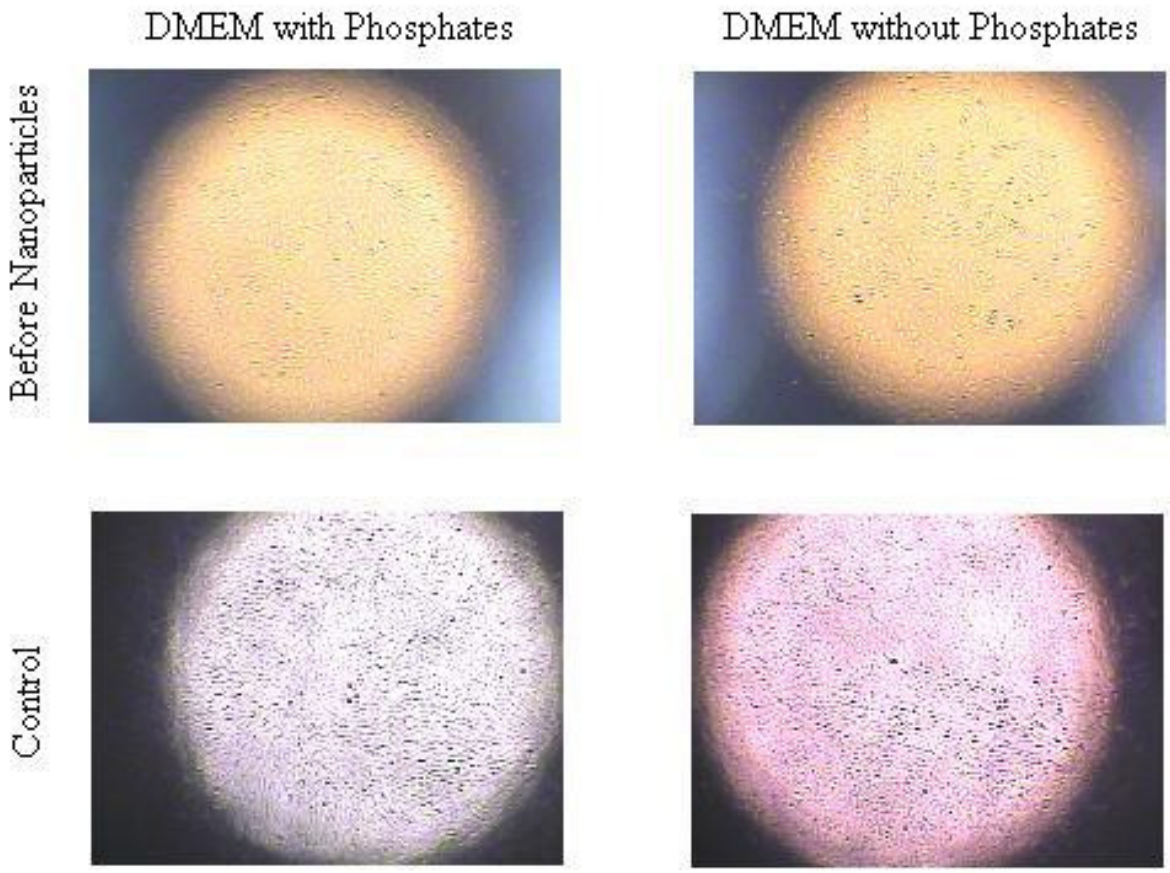
34. Chow , N.; Bell, R. D.; Deane, R.; Streb, J. W.; Chen, J.; Brooks, A.; Van Nostrand, W.; Milano, J. M.; Zlokovic, B. V. *PNAS* **2007**, *104*, 823-828.

35. Analysis performed on a PC computer using the public domain Scion Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://www.scioncorp.com/pages/download_now.asp)

36. Kirchner, C.; Munoz Javier, A.; Susha, A. S.; Rogach, A. L.; Kreft, O.; Sukhorukov, G. B.; Parak, W. J. *Talanta* **2005**, *67*, 486-491.

37. Gold, R.; Schmied, M.; Rothe, G.; Zischler, H.; Breitschopf, H.; Wekerle, H.; Lassmann, H. *The Journal of Histochemistry and Cytochemistry* **1993**, *41*, 1023-1030.

APPENDIX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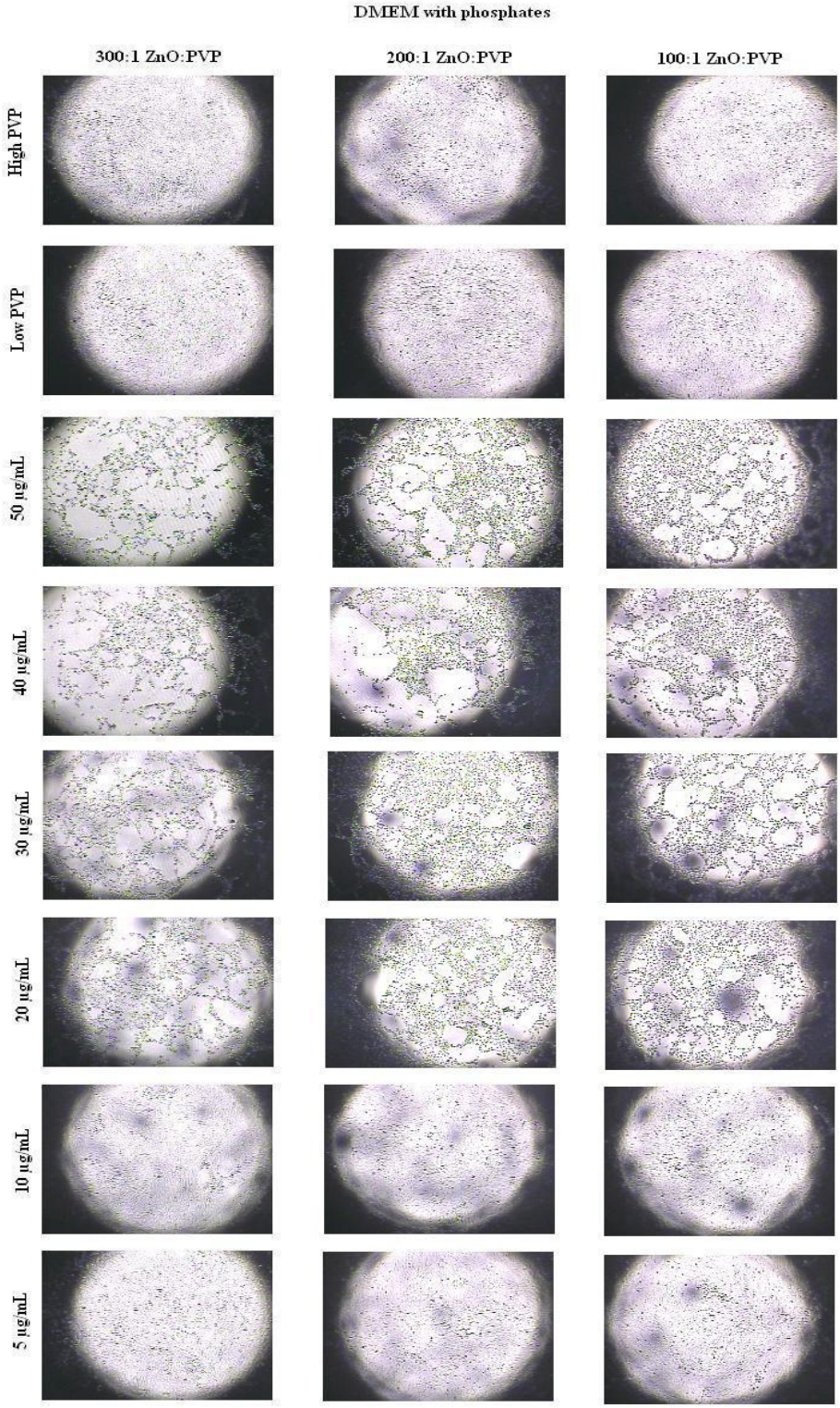
DMEM without Phosphates
Before Nanoparticles
Control


DMEM with phosphates
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200:1 ZnO:PVP
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High PVP
Low PVP
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40 µg/mL
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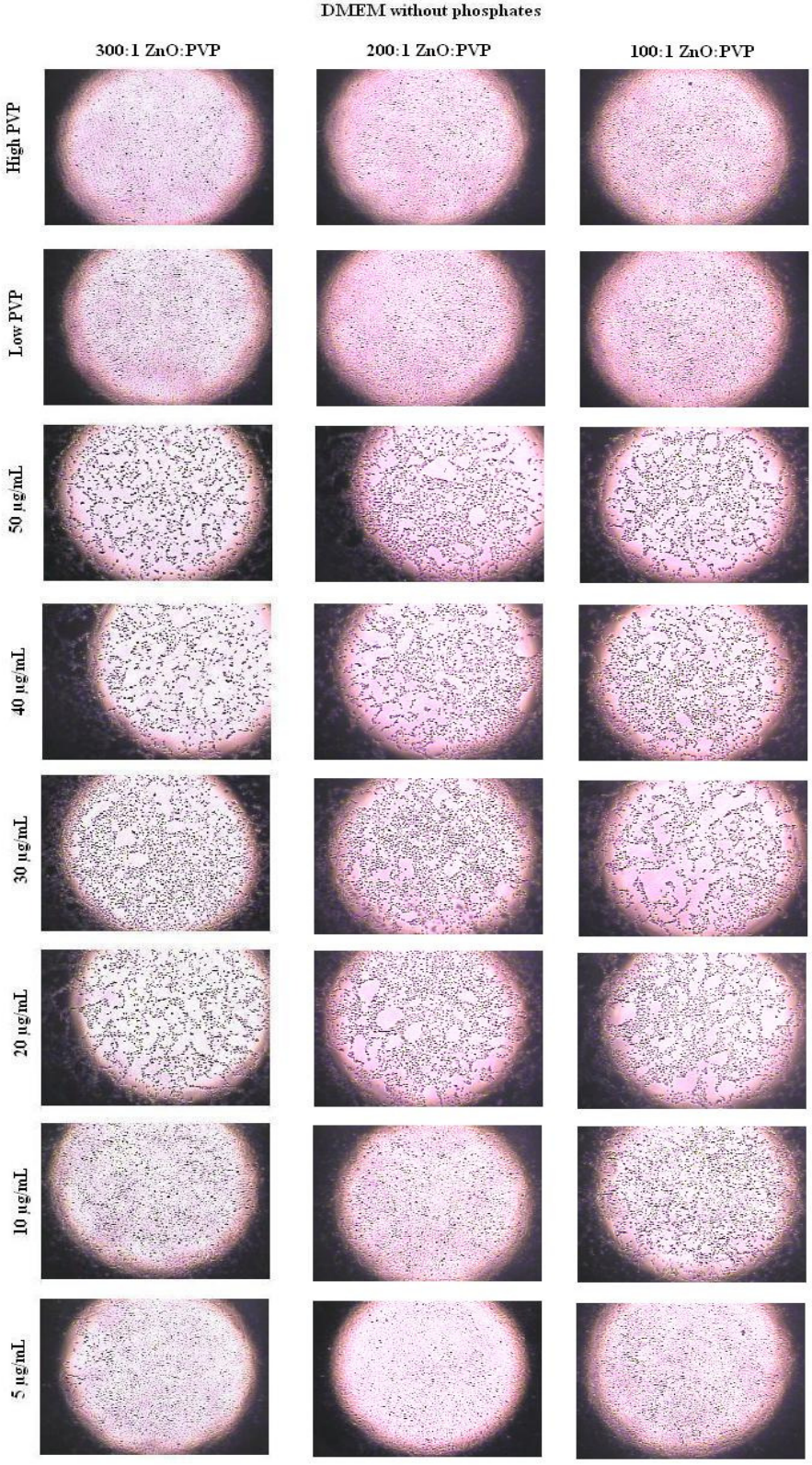
20 µg/mL
10 µg/mL
5 µg/mL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200:1 ZnO:PVP
100:1 ZnO:PVP
High PVP
Low PVP
50 µg/mL
40 µg/mL
30 µg/mL
20 µg/mL
10 µg/mL
5 µg/mL

RT-PCR results from 24 hours after 2-D transfection of siRNA

	CONTROL				TRANSFECTED			
	GAPDH	**GAPDH**	**SRF**	**SRF**	**GAPDH**	**GAPDH**	**SRF**	**SRF**
C_T	20.87	20.37	26.69	25.59	17.25	16.95	23.94	23.63
Mean C_T		20.62		26.14		17.1		23.785
ΔC_T[a]				5.52				6.685
$\Delta\Delta C_T$[b]				0				1.165
$2^{-\Delta\Delta CT}$				1				0.445964

[a] $\Delta C_T = C_T$ SRF- C_T GAPDH.
[b] $\Delta\Delta C_T = \Delta C_T$ SRF- ΔC_TSample

RT-PCR results from 48 hours after 2-D transfection of siRNA

	CONTROL				TRANSFECTED			
	GAPDH	**GAPDH**	**SRF**	**SRF**	**GAPDH**	**GAPDH**	**SRF**	**SRF**
C_T	33.62		34.95	36.95	23.97		24.32	23.96
Mean C_T		33.62		35.95		23.97		24.14
ΔC_T				2.33				0.17
$\Delta\Delta C_T$				0				-2.16
$2^{-\Delta\Delta CT}$				1				4.469149

RT-PCR results from 24 hours after 3-D transfection of plasmid DNA

	CONTROL				TRANSFECTED			
	GAPDH	**GAPDH**	**SRF**	**SRF**	**GAPDH**	**GAPDH**	**SRF**	**SRF**
C_T	22.43	22.69	29.54	29.96	21.92	21.94	28.91	29.46
Mean C_T		22.56		29.75		21.93		29.185
ΔC_T				7.19				7.255
$\Delta\Delta C_T$				0				0.065
$2^{-\Delta\Delta CT}$				1				0.955945

RT-PCR results from 168 hours after 3-D transfection of plasmid DNA

	CONTROL				TRANSFECTED			
	GAPDH	**GAPDH**	**SRF**	**SRF**	**GAPDH**	**GAPDH**	**SRF**	**SRF**
C_T	25.91	25.78	31.36	31.95	26.31	26.79	32	32.39
Mean C_T		25.845		31.655		26.55		32.195
ΔC_T				5.81				5.645
$\Delta \Delta C_T$				0				-0.165
$2^{-\Delta \Delta CT}$				1				1.121166

RT-PCR results from 48 hours after 3-D transfection of siRNA (no media change)

	CONTROL				TRANSFECTED			
	GAPDH	**GAPDH**	**SRF**	**SRF**	**GAPDH**	**GAPDH**	**SRF**	**SRF**
C_T	29.19	28.94	33.86	34.94	29.49	29.17	34.8	34.57
Mean C_T		29.065		34.4		29.33		34.685
ΔC_T				5.335				5.355
$\Delta \Delta C_T$				0				0.02
$2^{-\Delta \Delta CT}$				1				0.986233

Table 2. RNA Quantification for 2-D Transfection of SRF Plasmid DNA

	Control 24 hrs	**Transfected 24 hrs**	**Control 48 hrs**	**Transfected 48 hrs**	**Control 72 hrs**	**Transfected 72 hrs**
Reading	1756.3	1638.6	3662.2	3497.2	5973.4	6175.3

Table 3. RNA Quantification for 2-D Transfection of SRF siRNA after 24 Hrs

	Control 24 hrs	**Transfected 24 hrs**
Reading	499.14	878.97

Table 4. RNA Quantification for 2-D Transfection of SRF siRNA after 48 Hrs

	Control 48 hrs	**Transfected 48 hrs**
Reading	79.132	948.54

Table 5. RNA Quantification for 3-D Transfection of SRF Plasmid DNA

	Control 24 hrs	**Transfected 24 hrs**	**Control 168 hrs**	**Transfected 168 hrs**
Reading	185.083	204.954	123.967	113.556

Table 6. RNA Quantification for 3-D Transfection of SRF siRNA after 24 Hrs

	Control 24 hrs	**Transfected 24 hrs**	**Control 24 hrs no change**	**Transfected 24 hrs no change**
Reading	88.089	84.99	80.93	81.803

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