INVESTIGATION OF GENE DELIVERY VIA SILICON NANOTUBES USING THE REPORTER GENE EGFP

by

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INVESTIGATION OF GENE DELIVERY VIA SILICON NANOTUBES

USING THE REPORTER GENE EGFP

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ABSTRACT

With the rising prominence of nanotechnology, materials such as silicon nanotubes offer great potential in a wide variety of fields. On such a small scale, the physical and chemical properties of the constituent elements are dramatically changed. Our research investigates the potential of silicon nanotubes as gene delivery vectors. As a non-viral vector, many of the harmful and potentially fatal side effects from traditional gene therapy are absent. Offering a viable treatment plan for diseases with little to no current treatment, silicon nanotubes show great promise in the clinical setting. Our group sought to use a reporter gene, eGFP, linked to the silicon nanotubes. Cells that took up the nanotubes should express the GFP protein, resulting in a measurable green fluorescence. Our research has shown, using the fluorescent tag FITC, the nanotubes interact with 293 HEK cells. Further studies are underway to effect delivery of the eGFP gene to 293HEK cells and validate the use of silicon nanotubes as a gene delivery vector.

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INTRODUCTION

Nanotechnology is a quickly growing scientific field with the potential for new and exciting applications. Nanotechnology refers to working with atoms and molecules to improve on existing technologies or create new applications and materials. It is a wide ranging field, covering areas from chemistry to biology to physics. Applications such as improved batteries or super-conductive materials have already been discovered. As scientists improve the methods and procedures used to manipulate materials on such a small scale, new applications will be discovered and improved upon.

One rapidly expanding field for nanotechnology is the biological field, particularly medical applications. At such a small scale, nanomaterials have the potential to interact with individual cells in ways previously thought to be impossible. Nanomaterials can be created for specific purposes that have no macro scale counterpart.

Graphene, a nanomaterial, is quickly gaining attention in the physics community. Composed of carbon atoms, it is a very versatile material with applications from chemical sensors to transistors. It can be easily modified by electrical and magnetic fields. Plus, it is easily obtainable from graphite (Castro Neto 2009).

Another important nanomaterial is gold nanoparticles, also called gold colloids. Besides having unique conductive and physical properties, gold colloids have many applications in biological systems. Gold nanoparticles can be conjugated to oligonucleotides and used as biosensors, markers for gene expression, and

disease diagnosis tools. The particles can also be conjugated to peptides, lipids, and enzymes (Daniel 2004).

Silicon nanoparticles are a type of biologically important nanomaterials that offer great promise in treating various diseases. Silicon is the most abundant element in the earth's crust. On a nanoscale, silicon can be formed into porous nanoparticles for various applications. The nanoparticles can be created in different sizes and surface functionalized to increase the breadth of applications. The silicon can also be formed into other shapes, such as nanotubes.

Recently, silicon nanoparticles have been shown to have a wide variety of applications in biological systems. Scientists have been successful in conjugating DNA to nanoparticles and delivering them to individual plant cells. These cells then can express a protein that was either absent or defective. For example, the GFP gene was conjugated to mesoporous silica nanoparticles (Vallet-Regí 2004). Tobacco plants took up these particles and expressed the GFP protein. This offers great promise to non-viral gene delivery (Torney 2007). Silicon nanoparticles have also been shown to deliver genes to different parts of the brain *in vivo*. Neurons that have lost functionality often have no means to regenerate. In a particular study, mice were injected with organically modified silica nanoparticles conjugated to the eGFP gene. This gene was expressed in the mice's brain tissue. Also, organically modified silica nanoparticles were conjugated to the FGF receptor type 1 gene. When injected into mice brains, the gene was expressed and shown to affect the cell cycle of neurons in the brain. These findings offer hope for patients inflicted with neuromuscular and neurodegenerative diseases such as Alzheimer's (Bharali 2005).

Gene therapy offers great promise in treating genetic diseases. Many of these diseases have little to no current form of treatment. In traditional gene therapy, the patient has a defective or absent gene. The protein produced by this gene does not function properly, therefore causing the symptoms of the disease. Gene therapy aims to replace this defective gene. Most current gene therapy approaches use viruses as the delivery vector of choice. To be successful as a vector, the virus must have the proper tropism, so that the gene is delivered to the correct cells. The gene of interest is inserted into the genome of the virus, and the virus allowed to replicate. The virus may be further modified to prevent any side effects from interfering with the treatment, e.g., removal of virulence genes. The virus is then administered to the patient. Once in the patient, the virus will make it to the target cells and deliver its "payload." Depending on the type of viral vector used, the gene will be expressed either transiently or incorporate itself into the host permanently. Common viral vectors in trials today are Retroviruses and Adenoviruses.

While virus-mediated gene therapy may seem like an invaluable treatment option, it comes with its fair share of downsides. The vector is still a virus, so the body will treat it like an infection. The host's immune system may attack and destroy the viral vectors before they can deliver their payload. For genes that are going to be incorporated into the host's genome, they must be delivered to the correct location. If the gene incorporates in the wrong place, it may knock out a crucial gene. It could also incorporate itself in front of gene and serve as a strong promoter and overexpress the downstream gene. While too little of a gene is bad news, but too much of the protein can also have negative side effects. If somehow

the vector makes it to the germ cells, any changes could be passed onto the next generation. In addition to all these issues, finding a virus that only interacts with the cells of interest is no easy task. If the defective gene is needed in lung tissue, using a viral vector that targets multiple types of cells may produce unintended side effects for the patient.

Clearly, viral vectors come with their fair share of downsides. Non-viral vectors have been explored, but there still remains a large amount of research that must be put into these alternative methods. One method, the gene gun, involves shooting gold nanoparticles coated in naked DNA directly into the tissue of interest. This allows for very fine-tuned control of gene delivery. Another method involves creating liposomes, synthetic lipid-based vesicles with a gene "payload." These vesicles can deliver the gene without the immune system recognizing it as foreign. It is able to evade the immune system and deliver its gene (Niidome 2002).

The goal of our research is to investigate non-viral gene vectors, specifically silicon nanotubes, as possible vehicles for gene delivery. Using a reporter plasmid, cellular uptake of silicon nanoparticles can be quantified using fluorescent microscopy. Expression of the reporter gene, eGFP, not only supports the fact that the cells took up the DNA but also that the plasmid is being transcribed and translated. Successful expression of the reporter gene offers promise for the silicon nanotubes as non-viral gene vectors.

METHODS AND MATERIALS

Construction of Reporter Gene

The gene used was eGFP, enhanced green fluorescent protein. In the lab, eGFP was incorporated into the plasmid pcDNA3. Using the restriction enzymes NotI and XhoI obtained from NEB, both pcDNA and eFIRES were cut. Once cut, both samples were run on an agarose gel using gel electrophoresis. The eFIRES plasmid will serve as the vector, while the eGFP gene excised from the pcDNA3 plasmid served as the insert. Both vector and insert were excised from the gel to be purified. The samples were purified using GENECLEAN from MP Bio according to the manufacturer's protocol. Once purified, the eGFP insert was ligated with the cut eFIRES plasmid using T4 DNA ligase (New England Biolabs). The solution incubated at 15° C overnight and then moved to the freezer.

The plasmids were transformed into a strain of E. Coli bacteria. 200 µL of bacteria were added to a microfuge tube and put on ice. 20 µL of the plasmid solution was added to the microfuge tube and allowed to incubate on ice for 10 minutes. The tube was then put in a 42° C water bath for 90 seconds. After 90 seconds, the tube was put back on ice for 2 minutes. 1 mL of SOC medium (obtained from Sigma-Aldrich) was added to the tube. The tube was incubated at 37° C for 45 minutes. 100 µL of this solution was spread on an agar plate and allowed to incubate at 37 ° C overnight. The agar plate was treated with ampicillin. Bacteria that had taken up the plasmid were resistant to the ampicillin due to the ampicillin-resistance gene located on the eFIRES-p plasmid. Surviving colonies were grown up to 2 mL in a LB and ampicillin culture. From this culture, the miniprep protocol was

followed to extract the plasmid from the bacteria. 1.5 mL of the culture was added to a micro centrifuge tube and spun at 8000 rpm for 2 minutes. The supernatant was removed. 200 µL of Solution 1 (25 mM Tris-HCl pH8, 50 mM glucose, 10 mM EDTA, and 4 mg/mL lysozyme) was added to the tube and incubated at room temperature for 5 minutes. 400 µL of Solution 2 (0.2 M NaOH and 1% SDS) was added to the tube and incubated on ice for 5 minutes. 300 µL of 3 M NH_4OOCCH_3 was added to the tube and allowed to incubate for 10 minutes on ice. The tube was spun for 3 minutes at 10,000 RPM. The supernatant was moved to another tube where 500 µL of isopropanol was added. The tube was vortexed and incubated at room temperature for 10 minutes. The tube was spun at 13,000 RPM for 10 minutes. The supernatant was removed, and 100 µL of 70% ethanol was added. The tube was spun at 13,000 RPM for 5 minutes. The supernatant was removed, and the pellet was allowed to dry inverted on a tissue paper for 10 minutes at room temperature. 50 µL of water was added to resuspend the DNA. To ensure the vector had taken up the insert, the DNA was cut with XhoI and NotI. It was run on a 1% agarose gel using gel electrophoresis. Clones that were positive for insert were selected and a midiprep was performed using the Zyppy Plasmid Midiprep kit (Zymo Research.)

Cell Culture and DNA Transfection

The final step for the plasmid was testing the gene in a cell *in vitro*. Using HEK 293 cells and the Lyovec protocol, cells were transfected with the eFIRES plasmid with eGFP insert.

Preparation of Nanoparticles

Nanoparticles were attached to FTO glass after synthesis. Using a spatula, nanoparticles were scraped off the FTO glass into a 50 mL beaker. 5 mL of acetone was added to the beaker. The solution was sonicated for 30 seconds. 1% APTES ((3-Aminopropyl)triethoxysilane) was added to the solution. The solution was allowed to incubate overnight while covered. The solution was added to dialysis tubing and placed in a 1 L beaker of DI water. A stir bar was added to the beaker. After 2 hours, the water was replaced. The solution was then allowed to dialyze overnight. The solution was then transferred to centrifuge tubes. 0.014 g of FITC (Fluorescein isothiocyanate) obtained from Sigma Aldrich was added to 1 mL of water in a centrifuge tube. The tube was then vortexed for 30 seconds. In a separate tube, 100 µL of the nanotube/APTES solution was added to 100 µL of the FITC/water solution. The solution was incubated 4 ° C overnight while covered. Care was taken to minimize exposure of FITC to light sources. The solution was added to dialysis tubing and placed in a 1 L beaker of water. The solution was allowed to dialyze for 2 hours with slow stirring, and then the water was replaced. The mixture was allowed to dialyze overnight. In the morning, the water was replaced again. The mixture was allowed to dialyze overnight again. The solution was then placed into centrifuge tubes in the freezer.

Conjugation of DNA to Nanoparticles

To conjugate the reporter plasmid to nanoparticles, 100 µL nanotube/water solution was added to a microcentrifuge tube. The tube was spun down, and the supernatant was removed. In a sterile environment, 200 µL of complete medium

was added to the tube, and the nanotubes were resuspended via vortexing. 1 µg of reporter plasmid was added to the solution, and the solution was allowed to sit at room temperature for 30 minutes. The solution was then used to treat 293 HEK cells.

RESULTS

Construction of Reporter Gene

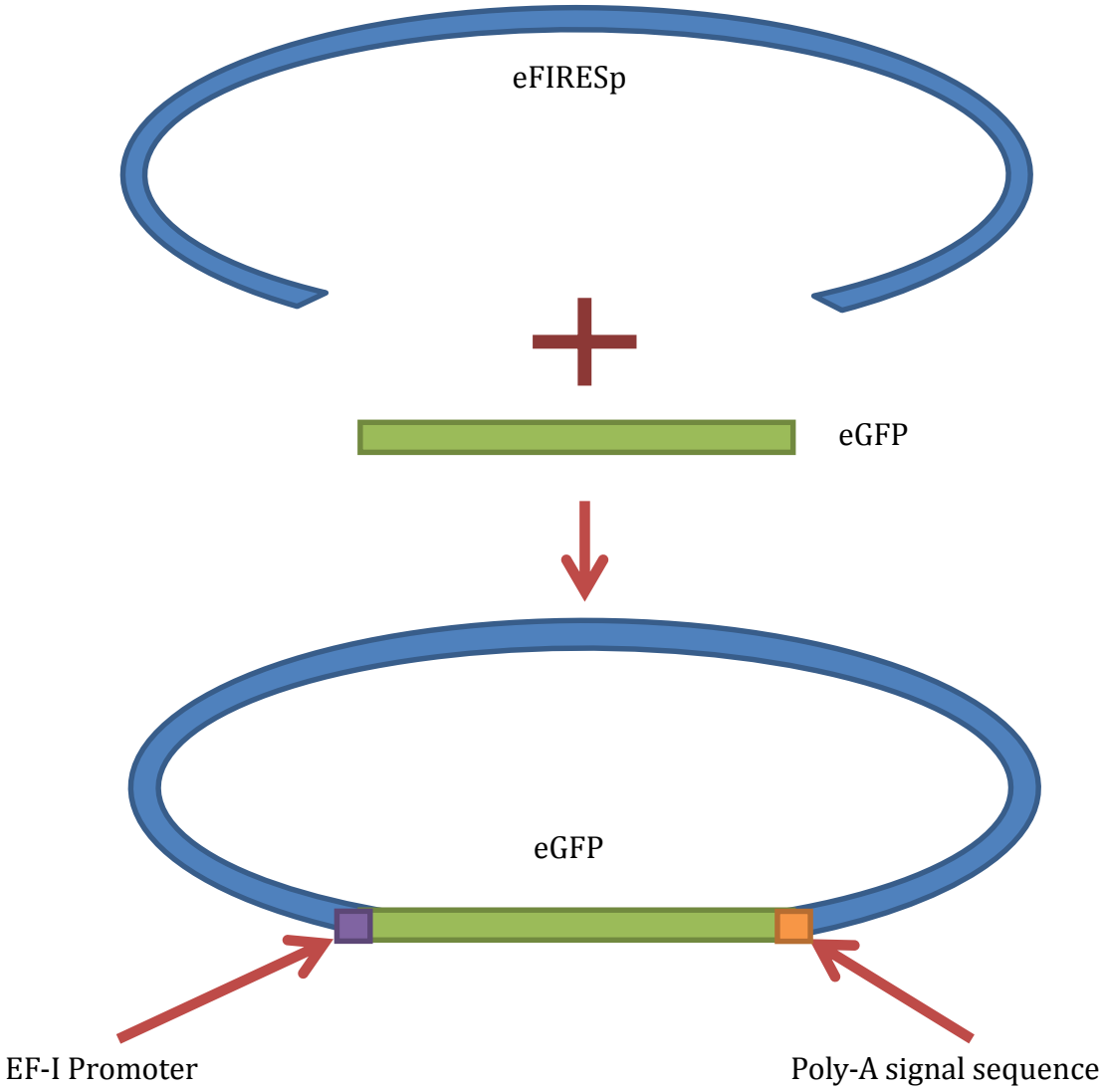
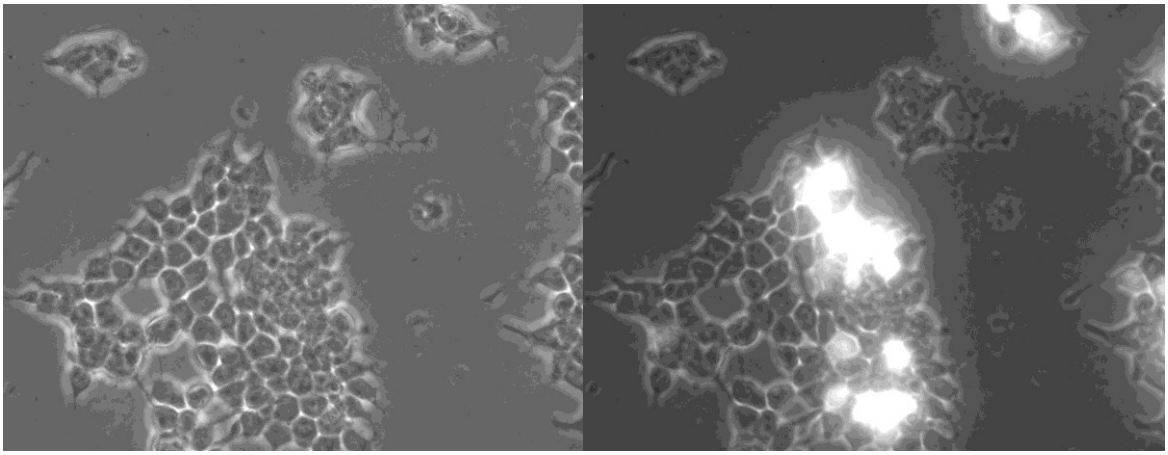


Figure 1: Ligation of vector and insert. The vector, plasmid eFIRESp, and insert, eGFP gene from pcDNA3 plasmid, were cut with restriction enzymes NotI and XhoI. Using T4 ligase, the vector and insert were ligated together. This plasmid will serve as the reporter plasmid.

To be able to assess the ability of the nanotubes to mediate gene delivery, a reporter plasmid had to first be made. The reporter we used was the enhanced Green Fluorescent Protein (eGFP). The eGFP gene was linked to a constitutive promoter, EF-1. If the DNA was able to enter the cell, the GFP gene would be transcribed and translated. The cell should turn green under the appropriate excitation wavelength.

Expression of Reporter Plasmid in 293 HEK cells

Once the reporter plasmid had been created, the plasmid needed to be tested to make sure that the gene could be transcribed and translated. It is not uncommon for mutations to be introduced into sequences during PCR, which makes this test important. 293 HEK cells were transfected with the reporter plasmid. After transfection, the cells appeared healthy under light microscopy. When viewed under fluorescent microscopy, cells that had taken up the reporter plasmid gave off a bright green fluorescence. In the images taken, around 24.8% of cells expressed the GFP gene. These results support the claim that the reporter gene was successfully inserted into vector.



Figures 2&3: Light microscopy and combination fluorescent/light microscopy of 293 HEK cells transfected with reporter plasmid.

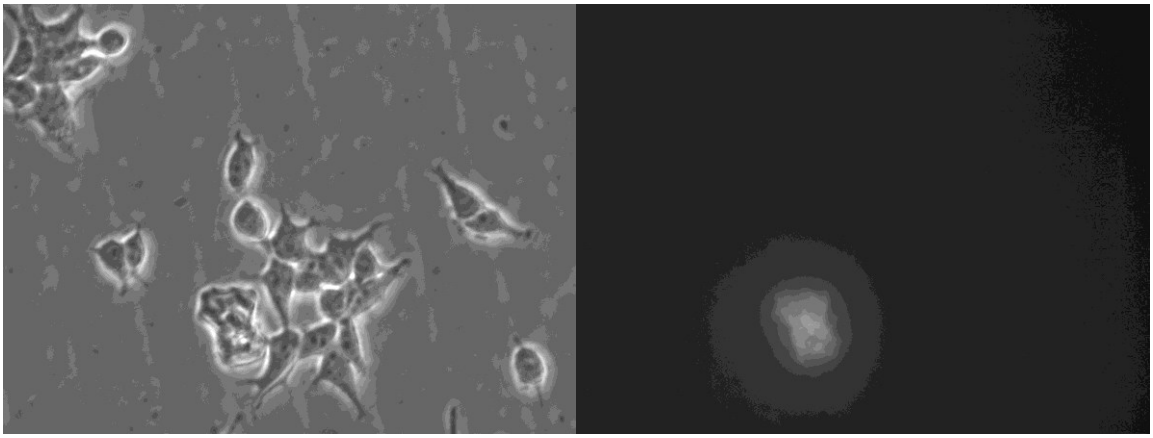
Conjugation to FITC

We first characterized the ability of the silicon nanotubes to attach to the cell with minimal cytotoxicity. To do this, we conjugated the nanotubes to FITC (a fluorophore) that would allow us to visualize the interaction between the cell and the nanotubes. Before nanotubes could be conjugated to FITC however, they first needed to be conjugated to APTES. APTES, or (3-Aminopropyl)triethoxysilane, is an aminosilane that can bond to the oxidized surface of the silicon nanotubes via covalent bonding. With a free amine functional group on the end of APTES, FITC can be bound to the negatively charged nitrogen. In later experiments, the positive phosphate backbone of DNA can be electrostatically bound to the free amine group. After the nanotubes had been conjugated to APTES, FITC needed to be conjugated to the APTES. This would serve as a fluorescent tag to observe the interaction of the nanotubes with the 293 HEK cells. Initial attempts at FITC conjugation resulted in excessive background fluorescence. The level of FITC in the wells was too high to view the cells under microscopy. We hypothesized that the traditional method of

treating with FITC, spinning down the nanotubes in a centrifuge, and discarding the supernatant was not completely removing the FITC. The residual FITC was then being transferred with the nanotubes to the cell culture. This excess FITC prevented the cells from being properly imaged.

To try and reduce the level of unbound FITC, the nanotubes were dialyzed after the conjugation step. The nanotubes were conjugated to FITC overnight and then transferred to dialysis tubing. This solution was allowed to dialyze in water overnight. Even after dialysis, the levels of fluorescence from FITC were too high and the cells were not visible under fluorescent microscopy.

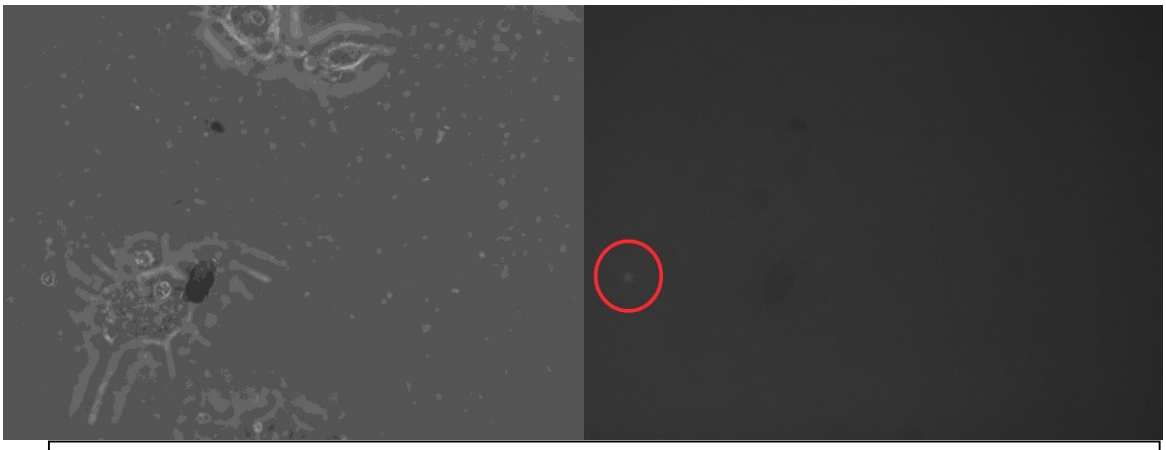
To further reduce the level of background fluorescence we next tried to reduce the amount of FITC used during conjugation with nanotubes. A study was conducted using normal strength (1.4 μg of FITC in 1 mL of water), 1:100, and 1:500 dilutions of normal strength FITC solution in water. After treating cells with the diluted solutions of FITC, the cells were able to be observed in the 1:100 dilution. The area corresponding to the cell membrane gave off a slight fluorescence. Under light microscopy, the cells appeared healthy. Aggregates of nanotubes could be seen in close proximity to the 293 HEK cells.



Figures 4&5: Light microscopy and fluorescent microscopy of 293 HEK cells treated with 1/100 FITC bound to silicon nanotubes functionalized with APTES.

DNA Conjugation

After observing interaction between FITC-bound nanotubes and 293 HEK cells, the next step was to determine whether the nanotubes could deliver DNA to the cells. Nanotubes functionalized with APTES were spun down, the supernatant removed, and the cells resuspended in cell culture medium. DNA was then added to the suspension for conjugation with the nanotubes. DNA-bound nanotubes were then added to 293 HEK cells



Figures 6&7: Light microscopy and fluorescent microscopy of 293 HEK cells treated with reporter plasmid bound to silicon nanotubes functionalized with APTES. Red circle highlights possible expression of GFP.

As seen in Figure 6, the silicon nanotubes appear to interact with the 293 HEK cells. While the cells appear to be relatively healthy, a few of the cells were surrounded by vesicles, a possible indicator of a form of programmed cell death known as apoptosis. When viewed under a fluorescent microscope, the vesicle near the cell interacting with the nanotube gave off a slight fluorescence. Similar instances of this phenomenon could be observed throughout the cell culture. Further investigation will need to be carried out to determine if GFP is being expressed, or if the cells are fluorescing due to apoptosis. This phenomenon, known as autoflourescence, can occur in cells during the early stages of apoptosis.

DISCUSSION

In this study, different molecular biology techniques were employed to test our hypothesis that silicon nanotubes could be used as gene delivery vectors. The first step in this project was to create a reporter plasmid, by excising the reporter gene, enhanced Green Fluorescent Protein, and inserting it into the vector, eFIRESp. This reporter gene would now be under the control of a constitutive promotor, EF-1, and, if delivered inside cells, would be transcribed and translated. The presence of green cells would be a rapid and conclusive indication of successful gene delivery. After growing colonies of E. Coli with the recombinant plasmid, plasmid DNA was harvested from the bacterial colonies. To confirm the presence of the GFP gene in eFIRESp, gel electrophoresis was performed on the plasmids harvested from the

colonies. The plasmids were cut with restriction enzymes XhoI and NotI and run alongside uncut vector and insert samples. The plasmids separated into two parts: one corresponding to the GFP gene insert and one corresponding to the eFIRESp vector. These results supported our claim that the GFP gene had been inserted into the eFIRESp vector.

The next step was ensuring that the plasmid could be expressed successfully in a mammalian cell line. Using 293 HEK cells and the Lyovec protocol, the plasmids were transfected into the cells. When exposed to the proper excitation wavelength, the cells gave off green fluorescence. This led us to conclude that the plasmid contained an intact reporter gene under control of the constitutive promoter. This plasmid could now be used in further experiments to assess gene delivery. If cells took up this plasmid, the GFP gene should be transcribed and translated, resulting in the green fluorescent protein.

With the reporter plasmid created, the "vehicle" for gene delivery had to be prepared. After removal from the FTO glass the nanotubes were fabricated on, they were sonicated and functionalized. The sonication ensured that the particles would not be too large and unable to interact with the cells. Functionalization would allow us to attach FITC and later DNA to the nanotubes. The linker used was APTES. APTES contains a free amine functional group which can bind to positively charged particles.

The rationale behind conjugating FITC to the nanotubes was to assess the interaction between the cells and nanotubes. If the nanotubes killed the cells or did not come in close proximity with the cells, their efficacy as a gene delivery vector

would be in question. FITC, a flourochrome, would give off fluorescence under the appropriate wavelength. Due to their conjugation to the nanotubes, the location of the nanotubes could be visualized using this method. FITC-conjugated nanotubes were dialyzed for purification from unconjugated fluorchrome. In addition, the FITC concentration used in conjugation with the nanotubes was reduced to decrease background fluorescence (caused by unconjugated FITC). The FITC-labeled silicon nanotubes could be visualized interacting with the cells. The nanotubes appeared to interact with the membrane of the cells. While it was impossible to determine if particles were entering the cells or just interacting with the cell membrane, it was clear that the nanotubes were in close proximity to the cells. The cells also remained healthy following treatment. These observations led us to conclude that the nanotubes were interacting with the cell membranes and possibly entering the cells and were exhibiting minimal cytotoxicity. These results allowed us to continue our investigation into nanotubes as gene delivery vectors.

The final experiment was a culmination of all previous steps. The reporter plasmid created earlier was conjugated to the APTES-nanotube complex. The cells were then treated with the nanotubes bound to DNA. Looking at the cells under the microscope, a few points of interest emerged. First, the cells in the experiment appeared healthy but were surrounded by vesicles in many cases. This may suggest that the cells were undergoing apoptosis, or programmed cell death. Second, some of these vesicles gave off slight fluorescence. This fluorescence may be caused by autoflouorescence, but it could also support the conclusion that the reporter genes were getting into the cell and being expressed. Finally, some large aggregates of

nanotubes were found in the cell culture. These aggregates were too large to enter the cell, so they would not have been able to properly deliver the reporter plasmid.

Overall, the results suggest that silicon nanotubes interact with 293 HEK cells. They can be found in close proximity to the cellular membrane. In regards to silicon nanotubes as gene delivery vectors, the results were inconclusive. While some vesicles gave off fluorescence, it was impossible to determine if the nanotube-DNA complex was responsible for this. Also, the cells did not fluoresce anywhere near the amount of fluorescence observed with the cells transfected by the reporter plasmid using the Lyovec protocol. A viable gene delivery vector would need to be efficient to warrant the treatment.

Moving forward, several steps could be taken to increase the efficacy of the nanotube gene delivery. First, a new set of 293 HEK cells can be used. The presence of sub-cell sized vesicles brings into question the health of the cells in the final experiment. A healthy set of cells would increase the likelihood of cellular uptake of the reporter plasmid. Second, the nanotubes could be sonicated before treating the cells. The large aggregates observed would be unable to deliver the reporter plasmid to the cells. Sonication would ensure the nanotubes were small enough to interface with the cells. Third, a greater amount of reporter plasmid could be used in the nanotube-DNA solution. This would ensure that more nanotubes were able to bind the DNA. Finally, the treated cells could be plated on a coverslip. After treatment, the coverslip could be mounted on a slide and observed on a microscope with higher resolution than the fluorescent microscope. This would allow us to observe the interaction between the nanotubes and cells in greater detail.

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