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Delivery of Antiviral siRNA with Gold Nanoparticle Against Dengue Virus

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Abstract

The goal of this study was to use specific small-interfering RNA (siRNA) sequences to target the *E* gene of the Dengue virus serotype 2 (DENV-2). It was predicted that this targeted binding of siRNA would prevent subsequent translation of the *E* gene protein, as well as virion production in infected cells. Due to the instability of the antiviral siRNA, the siRNA was conjugated to gold nano-particles (AuNP) in order to provide stability during delivery to the infected Vero cells. Cells were transfected with siRNA-AuNP complexes prior to DENV-2 infection. The siRNA-AuNP complexes tested included two different siRNA sequences (at varying concentrations) paired with positively, negatively, or neutrally charged nano-particles. In one experiment, the effectiveness of these complexes were tested in cells that were incubated for 48 hours post-infection, while in another experiment the cells were incubated for 72 hours post-infection. The inhibition of viral propagation in infected cells was analyzed using the quantitative polymerase chain reaction (QPCR). The QPCR results obtained were used to calculate the DENV-2 *E* gene to *β-actin* housekeeping gene ratio in order to determine effective viral inhibition in the infected cells. The results of the experiment showed decrease in viral propagation in infected cells with the use of the positively charged AuNP. There was consistent viral inhibition in both types of siRNA tested, especially within cell samples that were incubated for 72 hours post-infection (as compared to a 48-hour post-infection incubation). However, further experiments must be conducted in order to confirm efficacy of the anti-viral siRNA.

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C**hapter One -** Problem Statement

The purpose of this project is to utilize gold-nano-particles (AuNP) conjugated to small-interfering RNA (siRNA) that target the structural envelope (*E*) gene of Dengue virus serotype 2 (DENV-2), thereby preventing protein translation and virion assembly. Antiviral siRNA will be delivered into Vero cells (green monkey epithelial cells) by an AuNP carrier with subsequent infection of DENV-2 *in vitro*. We propose that the delivery of the antiviral siRNA to the infected cells will prevent the production of functional *E* gene translation into a functional protein that would decrease viral propagation within the cell.

Introduction

DENV is a single-stranded, positive-sense RNA virus belonging to the family Flaviviridae. DENV is also classified as an arbovirus, meaning its transmission to hosts is by the mosquito vectors, *Aedes albopictus* and *Aedes eqyptii* The virus is transmitted through the mosquito's saliva when taking a blood meal from a human host. Surface proteins on the virus bind to target cell receptors on various cell types within the host, whereby the virus is endocytosed into the cell's cytoplasm, where viral replication begins (1).

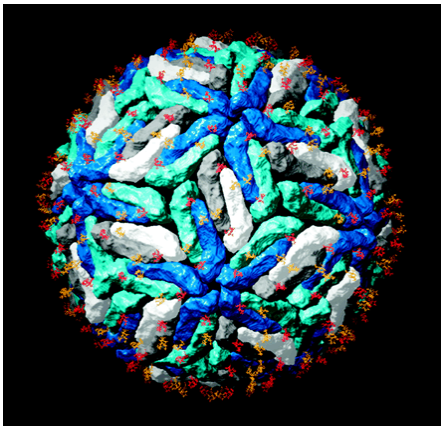


Figure 1: Surface/Structural proteins on Dengue virus. Envelope (E), membrane M and capsid (C) proteins. http://www.pnas.org/content/100/12/6899/F2.expansion.html#F1

DENV causes Dengue fever that is characterized by fever, headache, joint and muscle pain and a measle-like rash. Although many people infected with DENV fever fully recover, those cases which are severe have a significantly high mortality rate (1). Death due to DENV is usually the result of either Dengue shock syndrome or hemorrhagic fever. Dengue shock syndrome causes the body to go into a state of shock, resulting in dangerously low blood pressure and decreased heart rate. Hemorrhagic fever results in systemic leaking of blood from blood vessels and often results in death due to internal bleeding. There has been a significant increase in the number of cases of hemorrhagic fever occurring in Latin American countries over the past thirty years, especially in children. It is endemic in the tropics and subtropical regions of the globe where 40% of the population lives in areas at risk for transmission (1). There are no

current vaccines or antiviral drugs for the Dengue virus. In brief, development of therapeutics is pertinent to control infection.

Figure 2: Spread of Dengue hemorrhagic fever between 1981-2003. http://www.cdc.gov/dengue/epidemiology/index.html

The proposed method is to use RNA interference (RNAi), in particular siRNA to inhibit the expression of DENV-2 *E* gene, thereby preventing virion formation. siRNAs are naturally-occurring double-stranded RNA sequences that are formed to silence overexpressed mRNA that are induced in many pathological conditions. siRNAs work with several other cytoplasmic proteins that can target and silence viral RNA sequences that consequently prevent functional protein formation since silencing the gene of interest occurs post-transcriptionally. The size of siRNA sequences are usually 19-21 nucleotides in length and are formed as nascent duplexes that are further modified into single stranded RNAs by the RISC complex rendering them functionally able to specifically target and silence gene expression (Figure 3).

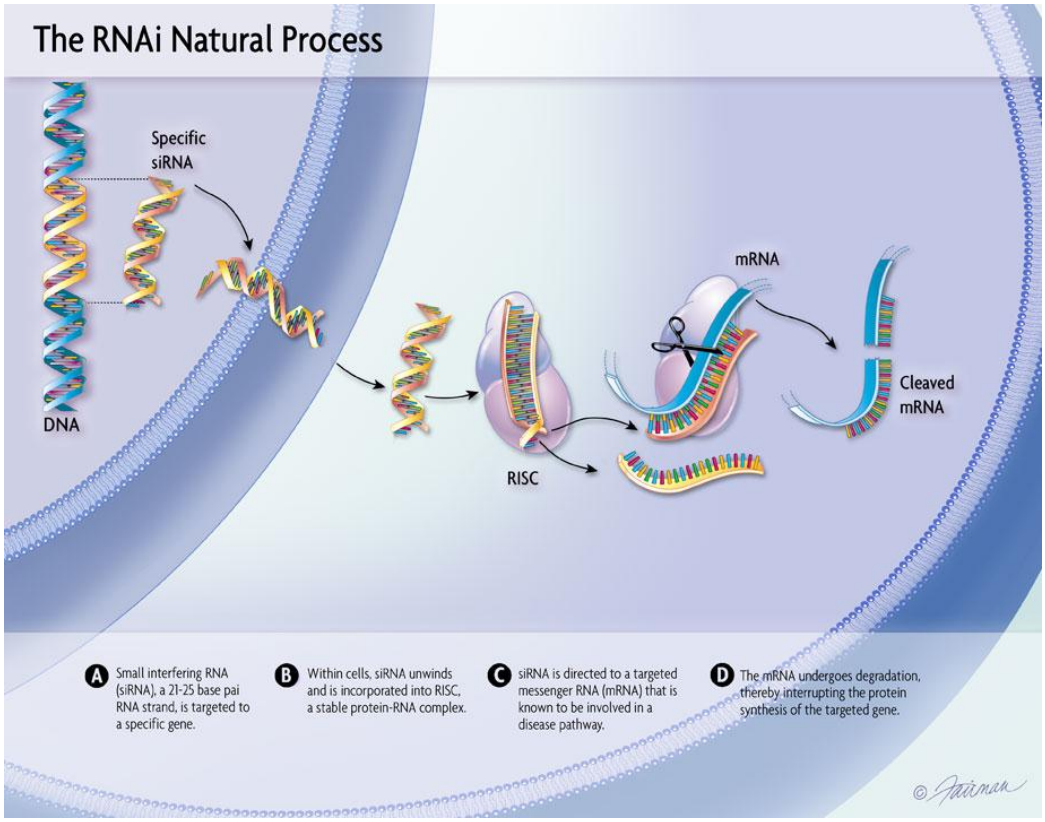


Figure 3: Formation of RISC-siRNA complex and subsequent mRNA targeting http://cantech.wpengine.netdna-cdn.com/wp-content/uploads/2012/05/rnai-interference.jpg

siRNAs specifically interfere with gene expression through its ability to change chromatin structure in the genome by working at the translational level. Synthetic antiviral siRNAs are often delivered to cells through the use transfection reagents or by way of electroportation. These antiviral siRNAs target specific viral RNA sequences, bind to them and either repress translation completely or cleave the viral RNA based

upon complementary of the siRNA that were designed (Figure 3). The use of siRNA is an important research tool for antiviral and therapeutic purposes.

The siRNAs have a characteristic structure, including 2 strands of a conserved length, each with predictable overhangs (Figure 4). It is believed that these overhangs are a determinant of the effectiveness of the siRNA. In a study designed to compare these determinant overhangs, it was shown that siRNAs with certain overhangs have a longer effective duration in cells, in comparison to siRNAs without that particular overhang (2).

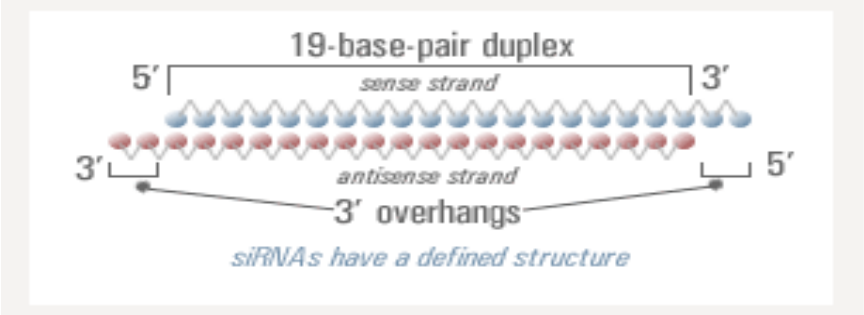


Figure 4: Basic siRNA structure, with characteristic determinant overhangs http://www.alnylam.com/rnai_primer/rna-interference-

The concern with using exogenous siRNA is that they are highly unstable and extremely sensitive to degradation by enzymes. Therefore a carrier is necessary for proper exogenous delivery of synthetically created siRNA in vivo. Our proposal

indicates that gold nanoparticles (AuNPs) are excellent carriers of siRNA that provide effective stability of the siRNAs in transport and prevents them from degradation.

In brief, specific antiviral siRNAs that target DENV-2 *E* gene expression conjugated to AuNPs can prevent virion formation in DENV- infected cells thereby, preventing further propagation of the virus *in vitro*. Using AuNP will not only allow for a more stable transport of the siRNA but will provide a system to deliver siRNA into cells without the use of a transfection reagent or electroporation. In our studies we tested two different siRNA models to inhibit DENV-infection of Vero cells. To determine that DENV was inhibited by siRNA-AuNPs, we compared viral copy expression in cells with siRNA-AuNPs coupled to a transfection reagent versus siRNA-AuNPs not coupled with a transfection reagent.

Chapter Two – Literature Review

The risk of DENV transmission has increased drastically within the last twenty years. There has been substantial research conducted on both the biology of DENV and possible mechanisms to inhibit viral propagation. Mosquitoes are a model organism to study DENV infection. A seminal paper by Xi et al., illustrated the basic pathway of DENV recognition in *Aedes* mosquitos. Briefly, the virus is transmitted to the mosquito by taking a blood meal from an infected mammalian host. Within the mosquito's gut tissue the virus replicates and disseminates to the salivary glands whereby the mosquito can transmit the virus to mammalian hosts during another blood meal (3). Within host cells, the entry pathway for DENV occurs through receptor-mediated endocytosis. A study by van der Schaar et al. in 2008 provided the evidence that DENV enters cells through endocytosis with a specific preference for clathrin-mediated endocytosis. Clathrin is a protein that forms endosome vesicles and binds DENV (4).

Previous research on DENV entry and its pathway to virion assembly was done by Alhoot et al. in 2011. The authors noted that understanding DENV entry is critical to limit replication and inhibiting binding of the virus on the cell surface would prevent the subsequent clathrin-mediated endocytosis of the virus. The authors identified the cell surface receptor (CD14) expressed on the surface of immune cells, as being the crucial receptor necessary for successful DENV entry (5). This study used exogenously introduced siRNA that targeted the CD14 receptor expression. CD14 targeted siRNAs silenced the translation of CD14 (85.2% reduction) and therefore no functional protein

was expressed on the cell surface (5). Thereby DENV binding was limited due to limiting the expression of CD14.

In terms of therapeutic use, many studies have utilized siRNAs to silence gene expression. For example, in a study done by de Paula et al., various siRNA segments were used that had been modified to improve their silencing efficiency. It is noteworthy that since exogenous siRNAs are "foreign" dsRNAs, unnecessary immunostimulation is possible. This study was able to design siRNA segments by chemically modifying siRNA segments to not only enhance repression of gene expression, but to reduce non-specific, immunogenic responses toward exogenously introduced siRNA (6). This study, in detail, concluded both chemical synthesis and *in vitro* transcription of siRNAs can specifically generate sequences that are functional. However, synthesized siRNAs are expensive, unstable in many conditions and sequences are fallible and potentially off-targeted if not developed correctly (6). Another method of siRNA generation involved endogeneous expression of siRNAs, whereby, transfected plasmids could encode the desired genetic information within the target cell. However, it is extremely difficult to introduce these siRNA encoding vectors into a cell.

As eluted to above, synthetic siRNA are unstable and subject to rapid degradation and therefore coupling siRNAs to a biocompatible molecule for stability has been a major research frontier. One study from Subramanya et al., examined the effects of siRNA delivery into dendritic cells infected with DENV (7). The method of siRNA delivery utilized in this study included a synthesized dendritic cell-targeting 12-mer peptide (DC3)

fused to nona-D-arginine (9dR) residues (DC3-9dR). When the siRNA-peptide complex was delivered into infected macrophages and dendritic cells, *in vitro*, the siRNA was able to target the DENV envelope (*E*) gene which successfully inhibited further replication of the virus (7). Furthermore, this method of delivery decreased cytokine production of lymphocytes, presumably due to reduced viral stimulation in the antigen presenting ability of macrophages and dendritic cells to lymphocytes (7). The authors also tested the effects of the siRNA complex *in vivo*. They designed a mouse model of DENV infection, whereby, they depleted the mouse of its immune cells and engrafted human stem cells with subsequent infection. They then treated the infected mice with the siRNA intravenously (7). This resulted in successful suppression of the DENV *E* gene and increased survivability of the infected mice (7).

Other methods of delivering siRNA have been proposed, including the use of gold nano-particles (AuNPs). AuNPs are an alternative method to siRNA delivery as they can efficiently transport siRNAs into cells, as well as, stabilize siRNAs. However, determining the cytotoxicity of the AuNPs is a recent challenge. A study done by Chen et al, used AuNPs to carry nucleic acids. Whereby, the AuNPs were removed from the final DNA-siRNA complex in order to prevent any negative cytotoxic effects that AuNPs may cause. The removal of any toxic threat is especially important because this delivery system was developed with the goal of silencing cancer-causing mRNA targets (8).

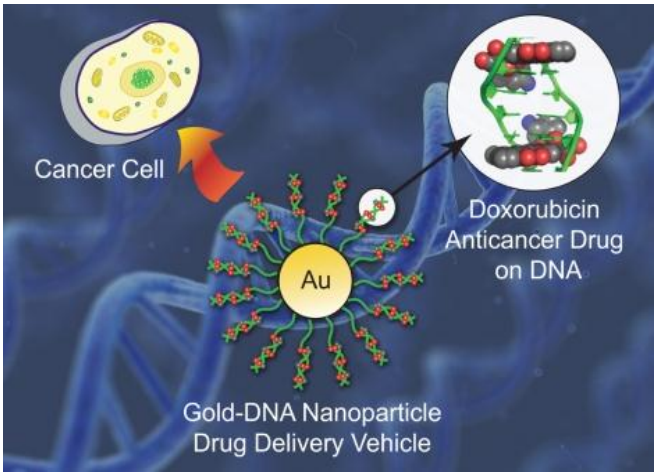


Figure 5: Gold nanoparticle used as a vehicle to stabilize the delivery of antiviral drugs. Chen, A. et al. 2012. *ACS Nano* 4.7: 3679-3688

The aforementioned studies describe key concepts of current studies being conducted with siRNA and AuNPs and furthermore, highlight important features to the development of a successful antiviral strategy. In this study, exogenously synthesized siRNA targeting the DENV-*E* sequence will be delivered into virally permissive cells by AuNPs. This strategy is intended to effectively silence expression of a functional envelope protein thereby inhibit the formation of DENV virions.

Chapter Three – Methodology

The purpose of this study is to silence viral gene expression to prevent further virion assembly through the use of siRNA and AuNPs, *in vitro*. In order to carry out these objectives, appropriate siRNA must first be synthesized. The siRNA synthesized for this study must match particular sequences of the DENV genome so that the siRNA can appropriately bind to and inhibit viral gene expression. The siRNA designed is complementary to the DENV envelope viral (*E*) RNA and was synthesized by the commercial source (Invitrogen). Following synthesis, the confirmation of sequence specificity was ensured using the website BLAST (Basic Local Alignment Search Tool). The three differential siRNA that were synthesized were tested in cell culture.

The DENV-infected cells that will be utilized in this study are Vero cells. Vero cells are a cell lineage isolated from African green monkey epithelial cells (9). Vero cells are used in many viral propagation studies as they are able to replicate continuously without become senescent and do not produce the anti-viral response, type I interferons that can inhibit viral replication (9). Vero cells were taken from liquid nitrogen stock and plated in DMEM media containing 20% fetal bovine serum. These cells were continually sub-cultured and monitored for confluence and viability. One week after bringing the Vero cells out of stock, the cells were transfected with two different siRNA sequences (siRNA-1 and siRNA-3). In carrying out the transfection, two plates were set-up: one plate containing samples with transfection reagent and one plate containing samples with no transfection reagent. The samples tested were Vero cells only, transfection reagent

only, a negative control transfer RNA (tRNA only-AuNP), siRNA-1 (si-1-AuNP) and siRNA-3 (si-3-AuNP) and a positive control DC-3-AuNP that has been previously reported to silence *E* gene transcription. Each sample was tested in triplicate. The tRNA, si-1, si-3, and DC-3 with AuNPs were added in 10 nM concentration of siRNA with an overall net positive charge of the AuNPs.

Thus, the Vero cells were transfected with siRNA alone or AuNP-siRNA with a transfection reagent, RNAi Max (Invitrogen). Following 48 hour incubation, these cells were infected with DENV-2 for an additional 72 hours. Post infection, vero cells were harvested and total RNA was extracted using TRIreagent (Molecular Research Center, Inc.). RNA was then converted to complementary DNA (cDNA) using the iSCRIPT reverse transcriptase enzyme and random primers (Bio-Rad). Following cDNA synthesis, specific primers and fluorescent probe designed for the DENV-2 *E* gene (Integrated DNA Technology and Applied Biosystems, respectively) were used for Q-PCR amplification along with the iTAQ supermix, containing polymerase (Bio-Rad).

As previously stated, the results of administering the synthesized siRNA to these infected cell lines was analyzed through the use of real-time polymerase chain reaction (RT-PCR) also known as quantitative polymerase chain reaction (Q-PCR). The difference between the commonly used method of PCR and Q-PCR is that in Q-PCR, the results of the reaction can be observed and recorded as they are happening (real-time), as opposed to the data being imaged at the end-point analysis by band comparison observed in an Ethidium Bromide gel. Q-PCR allows immediate and discernible analysis of the

amount of DNA amplified (produced) throughout the reaction (10). The samples tested will be compared to standard values in order to evaluate experimental DNA quantity using a standard curve.

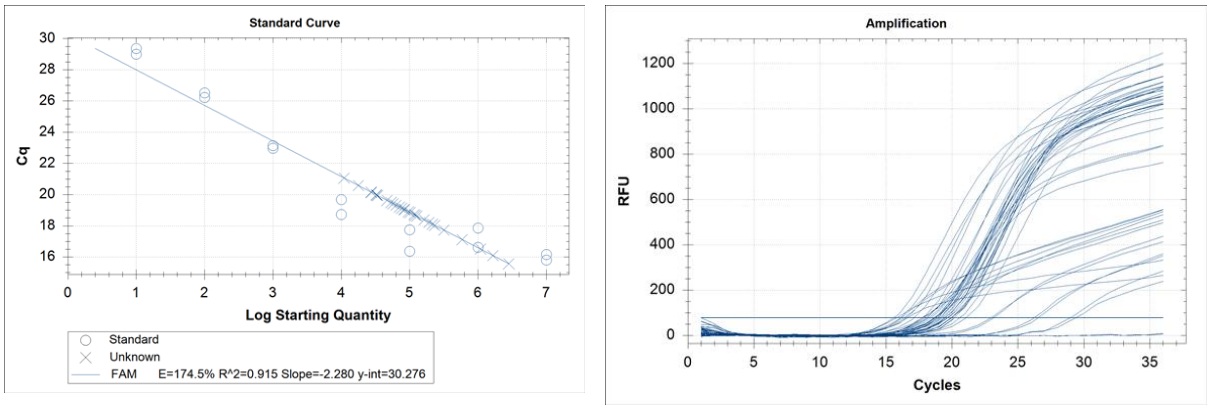
Following Q-PCR, the amount of DNA that is amplified can be quantitatively measured as a function of the amount of fluorescence emitted versus the number of cycles to amplification. The numerical amount of DNA that is generated (amplified) during the Q-PCR process makes it possible to analyze how much of the DENV-2 *E* is present in each sample. The experimental cell culture containing siRNA only, AuNP-siRNA with or without transfection reagent will be compared to a control sample in which only DENV was added (no siRNA). By comparing the number of DENV-*E* expression profile between the control and test samples can determine the effectiveness of the siRNA-AuNP delivery. After analyzing these quantitative results, modifications can be made to siRNA-AuNPs in order to improve inhibition of DENV-E.

Chapter Four – Results

Vero cells were kept in a continuous culture, and after siRNA-AuNP transfection, the cells were infected with DENV-2 (New Guinea C strain, ATCC). The viral media was removed 2 hours post-attachment and infection, and fresh media (DMEM supplemented with 10% FBS and 1% Penicillin/Streptomycin) was added and incubation continued for 72 hours. After incubation, the media was removed, and TRI reagent was added to the cells and total RNA was then isolated, and the concentrations of the RNA were measured using a Nano-Drop spectrometer. Based on the concentrations of the RNA, calculations were performed to prepare samples with a normalized concentration.

Following cDNA synthesis, Q-PCR standards for both DENV-2 *E* gene and *β-actin*, a housekeeping gene were prepared. The copy number for each standard was calculated and used to create a 10 fold dilution series. These standards were added in order to generate a standard curve to which the samples were compared. Based on the detected amplification of cDNA of differential samples, D-2 *E* to *β-actin* ratio was calculated and plotted (Figures 6, 7, & 8).

A



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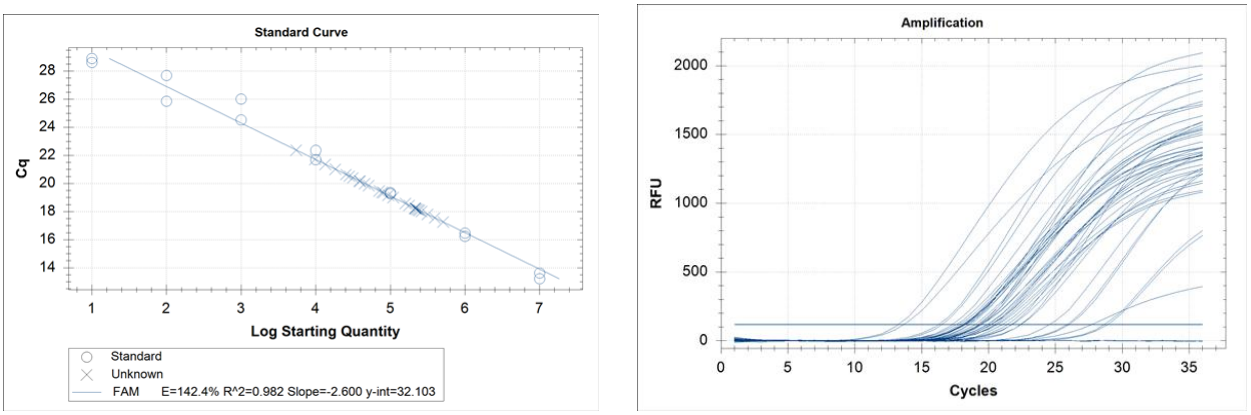

Figure 6. Standard curve and Amplification cycle plot for Q-PCR analysis. A. Standard curve and amplification plot for DENV-2 *E* gene. **B.** Standard curve and amplication plot for *β-Actin.* The Cq value (quantification cycle), which is more commonly known as the Ct (threshold value) describes the cycle number at which fluorescence increases above the threshold. When samples reach the threshold value, this indicates log amplification. The RFU value describes the relative fluorescence units.

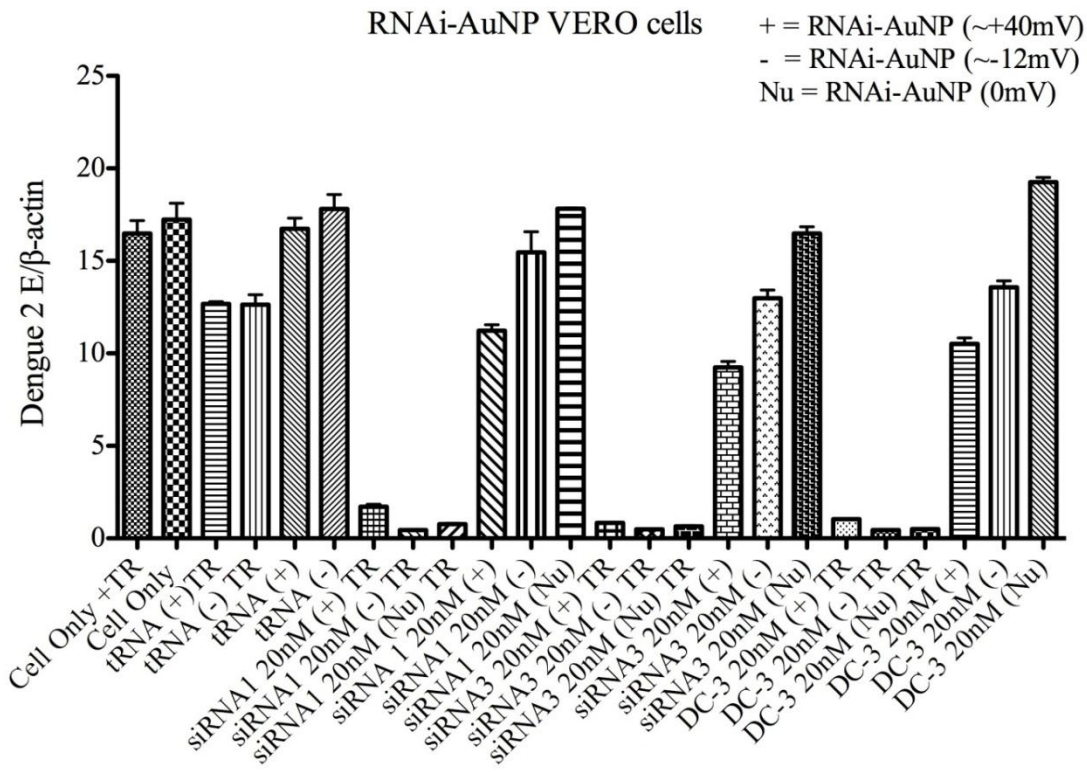

Figure 7. Ratio of DENV-2 *E* gene:*β-Actin*. The siRNA-conjugated to a positive or a negative charged AuNP to test the efficiency cell entry. All cells were incubated for 48 hours post-infection. DENV-2 *E* gene to β-actin ratios were compared. All samples were tested in triplicates. Both siRNA-1 and siRNA-3 (as well as the DC-3 positive control) were tested in 20 nM concentrations. The siRNA's and DC-3 were tested in six different ways: with a positively charged nano-particle (~40 mV), both with and without transfection reagent; with a negatively charged nano-particle (~-12 mV), both with and without transfection reagent; and with a neutral nano-particle (0 mV), both with and without transfection reagent.

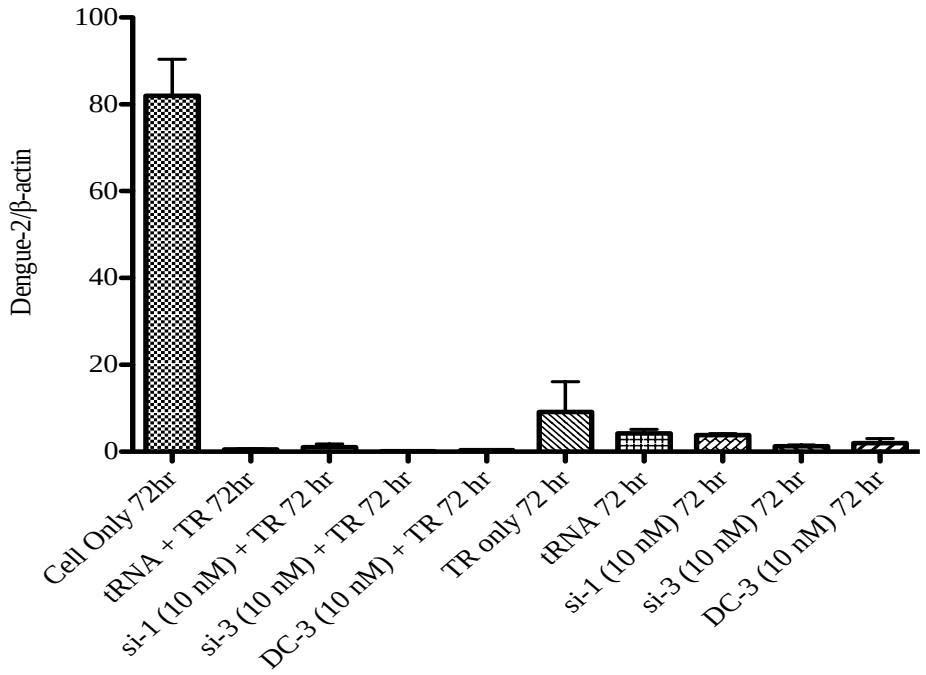

Figure 8. ***DENV-2 E : β-Actin*** **ratio in Vero cells**. Q-PCR analysis of Vero cells transfected with differential siRNA-AuNPs at 10nM. All samples are in triplicates.

Chapter 5 – Discussion

As previously stated, the goal of this study was to use siRNA conjugated with AuNP in order to target the *E* gene of DENV-2, thus effectively preventing translation of the protein and virion formation. The ultimate factor used to determine successful knockdown in this study was the ratio of DENV-2 *E* relative to the *β-actin* housekeeping gene. *β-actin* is used due to the fact that housekeeping genes are not generally changed by mechanisms such as anti-viral siRNA delivery unless cells are dying. Therefore, adequate levels of *β-actin* indicate viability of the cell's structure, meaning that the anti-viral techniques did not damage the cell in the process of inhibiting the virus. Based on the ratios of DENV-2 *E gene* to *β-actin* there were varying degrees of viral inhibition observed within infected cells. The level of inhibition was dependent upon the concentration of the siRNA, the charge of the nano-particle used and the length of incubation time post-infection. There was a notable difference in viral inhibition between the experiment involving cells that were incubated for 48 hours and 72 hours post-infection.

In the experiment involving cells incubated for 48 hours post-infection, the DENV- 2 *E* gene to *β-actin* ratio in cell only samples (with and without transfection reagent) were high as expected since DENV-2 E gene would be actively detected. The negative control tRNA without transfection reagent samples ratios were nearly equal with that of the cells only samples, indicating that the tRNA with a nano-particle alone was not effective in inhibiting replication.

The purpose of testing the samples with and without transfection reagent was to test the efficacy of the gold nano-particle to act solely as a transfection reagent. Both negative and positive nano-particles were used in order to compare their effectiveness in delivering the siRNA to the target gene. As the cell membrane is charged, it was important to test both types of nano-particles in order to determine whether a positive or negative charge was able to travel more successfully through the membrane. Typically, the positively charged nano-particles yielded lower *DENV-2 E : β-Actin* ratios, indicating the postively charged nanoparticle is more suited to cross the cell membrane (Figure 7).

Although the exact numbers varied slightly, the general trend among the siRNA's and DC-3 effectiveness were similar. In the samples containing transfection reagent (for siRNA-1, siRNA-3, and DC-3) there was significantly lower *DENV-2 E : β-Actin* ratios in comparison to the baseline cells only level (see Figure 7). This successful viral knock-down occurred with the use of all three types of AuNP. However, in the samples tested without transfection reagent (in which the nano-particle was acting as the transfecting factor), the amounts of viral inhibition were notably lower than in the samples tested with transfection reagent.

Furthermore, there were also appreciable differences in inhibition among the differentially charged nano-particles. In the case of the siRNA-1, siRNA-3, and DC-3, the neutral nano-particles yielded the least viral inhibition, with the *DENV-2 E : β-Actin* ratio nearly equal to the cells only ratio. The negatively charged nano-particles yielded slightly lower ratios, and the positively charged nano-particles yielded the lowest ratio levels

(Figure 7). Thus, in terms of using the nano-particles in place of a transfection reagent, the positively charged nano-particles were most effective in inhibiting viral replication. While the ratios of *DENV-2 E : β-Actin* using the positively charged nano-particles were not low enough to be considered a complete inhibitor of Dengue virus, these results do show that the positively charged nano-particles are the most effective in delivering the siRNA, as opposed to the neutral or negatively charged particles. Overall, these results supported further possible testing with postively charged AuNPs.

Thus, in the experiment involving cells incubated for 72 hours post-infection, the samples tested were each conjugated with a positively charged nano-particle only (excluding the cells only sample). In the sample containing cells only, there was a high level of *DENV-2 E : β-Actin* ratio, as expected. In the samples containing siRNA-1, siRNA-3, and DC-3 (each in 10 nM concentration) with no transfection reagent, there was a significant decrease in viral inhibition In these similar samples (with transfection reagent) the *DENV-2 E : β-Actin* ratio as close to 0, which would indicate total viral knock-down (Figure 8).

However, it is to be noted that the validity of the experimental results obtained from the 72 hour post-infection cells is questionable. While there was a significant amount of viral inhibition seen in this experiment (especially in the samples containing transfection reagent) this extreme knock-down may be adversely related to the incubation time. Having the cells incubate for 72 hours after initial infection (as opposed to 48 hours) may have caused damage to the cells, explaining the almost non-existent levels of

virus in the final *DENV-2 E : β-Actin* ratio analysis. If the cells' integrity was not maintained throughout the entire experiment, then the validity of the results is obviously subject to question of accuracy. However, additional experiments would need to be carried out in order to determine if the longer incubation time is a damaging agent for the cells.

In terms of future studies, there are still several options to be explored. Altering the charge of the nanoparticle could potentially increase the stability of the siRNA and ensure its targeted delivery to the virus. The charge of the nanoparticle is vital in that it delivers the siRNA through the lipid membrane (which contains both hydrophobic and hydrophilic regions). The most successful inhibition was seen using a positively charged nanoparticle (as opposed to negatively charged or neutral nanoparticles). Thus, in future studies the charge of the nanoparticle may be altered during synthesis of the particle in order to produce an even more positively charged particle in order to increase successful delivery of the siRNA. However, in the case of this study, our original hypothesis was supported in terms of the observed decrease in viral propagation in Dengue-infected cells, as a direct result of the delivery of antiviral siRNA conjugated to positively charged AuNP.

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