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Developing an Intranasal HIV-1 mRNA Vaccine Using Short Carbon Nanotubes (CNTs) as a Delivery System

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Abstract

Human immunodeficiency virus 1 (HIV-1) is a virus that attacks the immune system by destroying CD4 T cells, rendering them ineffective in protecting the body.(1) Despite 40 years of research, there are no viable HIV-1 vaccines currently on the market. In the paper, we performed experiments aimed to develop an effective needle free intranasal HIV-1 vaccine candidate. We optimized the ratio of antigen on the delivery vehicle carbon nanotubes (CNTs) and tested the effectiveness and toxicity of the vaccine formulations through *in-vitro* studies. The target antigen is mRNA encoding the HIV-1 first and second variable regions (V1V2) of glycoprotein gp120. To accomplish this goal, CNTs were sized and functionalized with polyethyleneimine (PEI) and 1,2-Dioleoyl-sn-glycerol-3-EPC (EPC) for optimal interaction with Calu-3 epithelial cells as the intranasal/mucosal delivery model. We conjugated mRNA that encodes enhanced green fluorescent protein (EGFP) as a model at varying ratios and tested levels of cytotoxicity, cellular uptake, and *in-vitro* translation. We incorporated surface functionalization molecules to stabilize mRNA on the CNT vehicle. The vaccine formulation that resulted in optimal mRNA loading, transfection, and *in-vitro* translation with the lowest level of cytotoxicity is the 5:1:2 CNT:mRNA:EPC formulation with CNT concentrations between 1 and 100 ug/mL.

Keywords: Carbon Nanotubes, HIV-1, mRNA vaccine, *in-vitro* studies

Introduction

In 2022, there were 650,000 deaths from HIV related causes and 39 million people living with the disease globally with an incidence of 1.3 million people.(2) While the annual incidence represents a 38% decrease from 2010, in which 2.1 million people acquired HIV, these statistics indicate the need for an effective vaccine for HIV. Currently, there is no such vaccine from conventional vaccine delivery methods. HIV-1 has a strong propensity for rapid mutation and sequence variation (rich biodiversity), which allows the virus to dynamically evade the immune system, posing major challenges to the development of a successful vaccine with a robust immune response.(3) The HIV-1 virion contains envelope proteins (Env), which are surface glycoproteins, specifically gp120 and gp41, that bind to CD4 receptors on T cells for cellular entry. Antibodies target epitopes on these glycoproteins to neutralize or eliminate the virion. However, the virus evolves to evade immune detection by varying its Env sequence, specifically in the V1V2 variable regions of the gp120 glycoprotein.(4)

Conventional vaccine types such as live-attenuated vaccines and inactivated vaccines result in ineffective methods of preventing HIV-1 through narrow immune responses that do not address the high variability of the virus. Live-attenuated vaccines are created by live viruses that are weakened which create a response by replicating in the body. Due to the variation of the HIV-1 antigens, the human immune system cannot elicit a broad enough antibody response using live-attenuated vaccines.(5) Another conventional method, inactivated vaccines, are not live and cannot cause the disease. However, they elicit a weaker and shorter-lasting immune response than live-attenuated vaccines and mRNA vaccines and therefore require multiple doses.(6)

Secondly, live-attenuated viruses risk the ability to retain viral properties and cause severe infections in patients with a weakened immune system as in HIV-1.(6) Additionally, latent viral reservoirs allow infected immune cells to remain in the body undetected by the immune system until they are activated by external factors and revive the virus in the patient.(7) Live-attenuated vaccines are unsafe because HIV-1 is a life-long infection, as opposed to other viruses, such as influenza. This puts people at risk of acquiring HIV-1, resulting in a poorer quality of life.

In recent years, mRNA vaccines became a viable alternative to these conventional methods for HIV-1. There are several advantages for using mRNA technology in this application. First, the body already has the machinery to translate mRNA into the glycoprotein antigens that are targeted by the immune system. The immune system is divided into two systems: innate and adaptive immunity. Innate immunity refers to the first line of defense and targets any foreign material in the body, making it nonspecific. Adaptive immunity takes over if the innate immune system cannot eradicate the substance and it takes on a more specific response through antibodies.(8) Research shows that proper control over HIV-1 in the body requires targeting not only the antibodies but also the innate immunity through CD8+ T-cells. mRNA has the ability to activate both immune systems in a balance to create a safe and effective response. While subunit vaccines containing mRNA are safer, they are less immunogenic than live attenuated vaccines, and therefore require a delivery system to prolong lifespan within the body and increase the cellular uptake efficiency. Lipid nanoparticles (LNPs) were used as delivery vehicles to create the current mRNA COVID-19 vaccine; however, these must be stored and traveled at ultralow temperatures (cold chain), giving rise to a need for a more easily stored and transported vaccine delivery method.(9)

Short CNTs are a novel vaccine delivery approach being developed at Luna Labs for mRNA vaccines that addresses the issues of conventional vaccines. CNTs are nanoscale materials made up of carbon from graphite sheets and are rolled into hexagonal mesh structures that can be layered into single-walled (SWCNTs) or multi-walled carbon nanotubes (MWCNTs).(10) CNTs have strong covalent bonds that hold uniform carbon atoms together. Therefore, it is highly stable in the body and on the shelf for a longer period of time than conventional vaccines.(11) The CNTs effectively deliver the antigens to the major histocompatibility

complex (MHCs) on antigen-presenting cells (APCs) of the immune system to launch a potent innate immune response. They can be made insoluble which keeps them in the immune system for longer and they also can hold many antigens on their surface to maximize exposure of the immune system to the antigen. The structure of CNTs allows surface functionalization with many molecules of high affinity to modulate immune responses within the body.[12] The nanoscopic size of CNTs also facilitates advantageous interactions with the cells in the body. The strong immune response and stability in the body removes the need for boosters. While the functionalized CNTs can create a strong immune response through these properties, the CNTs themselves are non-toxic to the body and not inherently immunogenic. Therefore, this eliminates the adverse and toxic side effects of vaccines that are seen with conventional vaccines.[11]

Current vaccines are injected intramuscularly; however, this method of vaccination does not provide a sufficient local immune response, such as the production of immunoglobulin A (IgA), an antibody found in mucosal membranes that is important in preventing HIV-1 infection. Intranasal vaccines have risen in popularity due to several advancements, including non-invasive injection and elicitation of systemic and mucosal immune responses. These vaccines target specific mucosal sites which are often the HIV-1 entry sites, providing immunity and reducing systemic infection. Additionally, the mucosal immunity that is provided by intranasal vaccination can catch the infection early to elicit front line immunity.[13] Not only do intranasal vaccines provide more localized immune responses, they also eliminate risks associated with needles contaminated with blood-borne pathogens.[14]

The proposed strategy utilizes polyethylenimine (PEI)-conjugated shortened CNTs as a vehicle that can be loaded with mRNA that encodes the V1V2 glycoprotein antigen. PEI is a cationic functionalization that allows for negatively charged mRNA to be loaded on the CNTs. This vaccine delivery system mimics the size and morphology of the HIV-1 virion and takes advantage of intranasal administration to provide frontline immunity at mucosal sites within the body. By mimicking the HIV virus, the cells are able to interact with the vaccine in a more natural way. This is hypothesized to increase the uptake of the vaccine by the cells. To incorporate the intranasal aspect of the vaccine, the transfection and cytotoxicity tests are conducted in Calu-3 cells. Calu-3 cells are an epithelial submucosal gland cell line.[13] This delivery approach improves upon conventional methods through its safe and non-invasive delivery system that targets local and systemic immune responses.

Results

Preparation of Short CNT Vaccine Formulation

PEI Conjugation to Short CNTs

Polyethylenimine (PEI), a cationic polymer, was conjugated to short CNTs within a size range of 100 to 300 nm and charge range of -20 to -30 mV to make the goal size and charge of the resulting CNT-PEI of 300 to 500 nm and +20 to 40 mV. Dynamic light scattering (DLS) was used to determine the average size of the CNT-PEI molecules to be 356.6 ± 57.72 nm and Zeta Potential Analysis was used to determine that their charge was +47.5 ± 16.3 mV as seen in Figure 1. Despite this charge not being in the exact range desired, the increased cationic character of the CNT-PEI will aid in facilitating anionic mRNA loading onto cationic CNT-PEI. At this point, all CNT samples used were conjugated with PEI, making CNT-PEI, but will be referred to as CNT.

mRNA:Short CNT Bonding Affinity

Gel electrophoresis was performed to determine bonding affinity of mRNA to short CNT. Samples with the best binding will not have a band

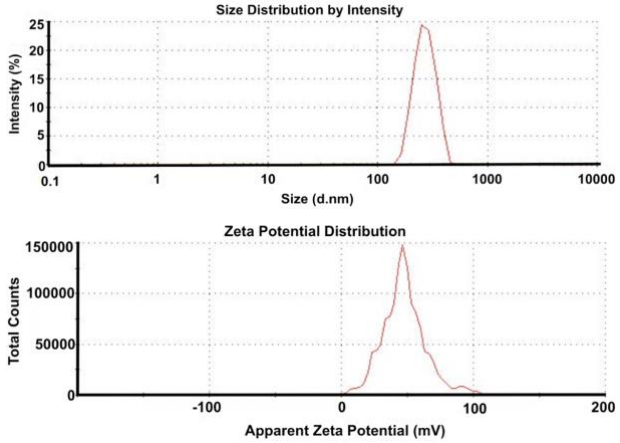

Fig. 1. Size and charge measurements of short CNTs analyzed through Dynamic Light Scattering after cutting and PEI conjugation.

on the gel as all of the mRNA will be bound to the short CNT in the well. In the first run, CNT:mRNA ratios of 0:1, 1:5, 1:1, 1:2, 2:1, and 5:1 was run; however intense bands were present in all lanes, except for a fainter band present in the 5:1 lane. Additionally, there were CNTs observed in the wells containing the following ratios: 1:1, 2:1, 5:1 CNT:mRNA. The experiment was repeated with new ratios containing higher amounts of CNT, as shown in Figure 2. Fainter bands are present for all lanes, with the best binding present in the 10:1 CNT:mRNA ratio as shown by the absence of a band in lane 9. CNTs were observed in all the sample wells. Ratios containing high concentrations of CNT were still not optimal, so a cationic esterized phospholipid, 1,2-Dioleoyl-sn-glycerol-3-EPC (EPC), was introduced to increase bonding affinity of mRNA to CNT.

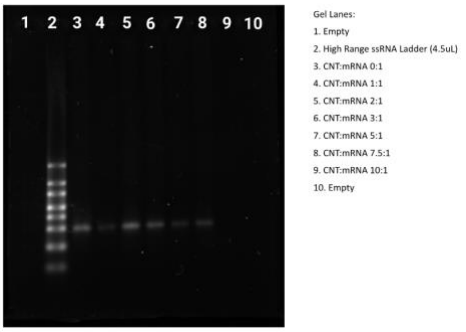

Fig. 2. Gel electrophoresis measuring the bonding efficiency of mRNA comparing different ratios.

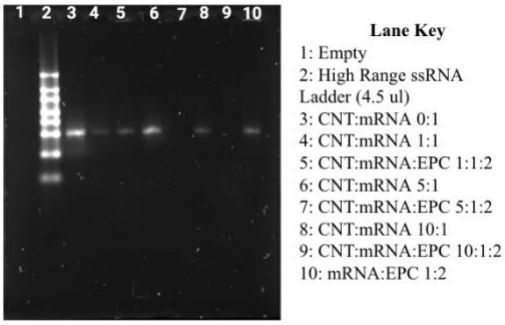

Fig. 3. Gel electrophoresis measuring the bonding efficiency of mRNA comparing different ratios with and without EPC

EPC, in combination with PEI, can stabilize and protect the mRNA loaded onto the CNTs. A final run of gel electrophoresis was performed containing samples with and without EPC, as shown in Figure 3. mRNA bands were present for all samples, except for the 5:1:2 and 10:1:2 CNT:mRNA:EPC samples, indicating that EPC enhanced the binding of mRNA to CNT in this formulation.

mRNA Loading Efficiency

Loading efficiency of mRNA was measured using fluorescence plate analysis on samples containing CNT:mRNA:EPC ratios of 5:1:0, 5:1:2, 1:1:0, 1:1:2, 0:1:0, and 0:1:2. The loading efficiencies of the 5:1 and 1:1 CNT:mRNA samples had loading efficiencies of 23.37% and 2.32%, respectively, while the loading efficiencies of the 5:1:2 and 1:1:2 CNT:mRNA:EPC samples had loading efficiencies of 82.66% and 37.08%, respectively. These results, visualized in Figure 4, indicate that incorporating EPC allows for increasing loading efficiency of mRNA to CNT, with the 5:1 ratio resulting in the best loading efficiency.

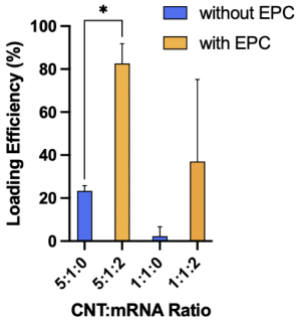

Fig. 4. Loading efficiencies of mRNA bound to CNT at CNT:mRNA:EPC ratios of 5:1:0, 5:1:2, 1:1:0, and 1:1:2.

Testing of formulations

Cytotoxicity of surface functionalized CNTs using a lactate dehydrogenase (LDH) assay

As the interest of this study is to investigate the use of the CNT-PEI formulation with and without EPC for biological use, the goal of the LDH assay was to determine the toxicity of this formulation without mRNA. Additionally, since EGFP mRNA was being utilized, including it in this study would not provide insight into the cytotoxicity of the actual formulation once V1V2 mRNA was used. The CNT formulations were prepared with and without EPC at concentrations of 500 ug/mL, 100 ug/mL, 10 ug/mL, and 1 ug/mL. They were tested against a cells only

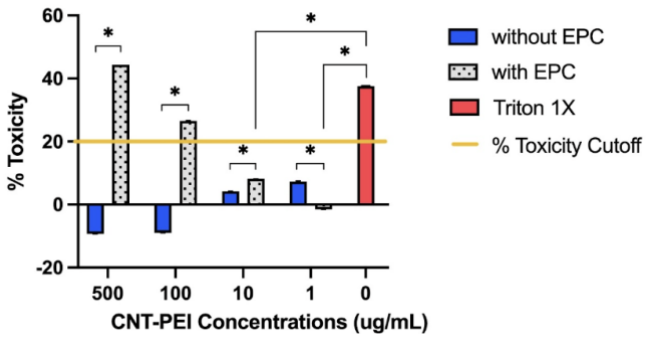

Fig. 5. LDH Assay comparing % toxicity of different concentrations of CNT-PEI with and without EPC as well as a positive control of Triton 1X. The toxicity cutoff is indicated at 20% with a yellow line.

negative control and Triton 1X positive control. A toxicity cutoff of 20% toxicity was pre-determined from literature.[15] Percent toxicity is defined as the percentage of dead cells in the well. Figure 5 shows the results of the LDH assay. All the samples without EPC were viable while the 10 ug/mL and 1 ug/mL CNT-PEI concentrations with EPC were viable.

In-Vitro Translation Assay

An *in-vitro* translation assay was performed to compare the level of translation in the Calu-3 cells of mRNA alone and mRNA attached to CNT-PEI. The mRNA on the CNTs was translated to a concentration of

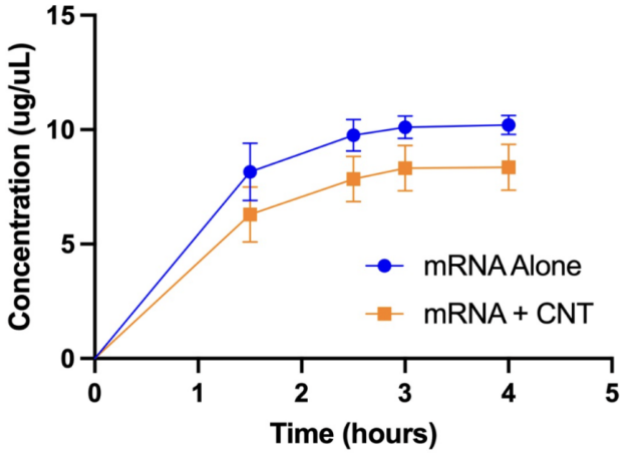

Fig. 6. IVT assay comparing *in-vitro* translation of mRNA alone and mRNA attached to CNT-PEI in Hela cell lysate.

81.9% of the amount from mRNA alone. Both curves followed the expected path as they both grew in hours 1 and 2 and then plateaued between hours 3 and 4. Statistical analysis gave a p-value of 0.1376 and showed that there was no significant difference between the translation of mRNA alone and the mRNA loaded on the CNTs. A second IVT was performed but it was not presented because of errors mentioned in the discussion. A third IVT was performed comparing 5:1:2 and 10:1:2 CNT:mRNA:EPC ratios with an mRNA only control, seen in Figure 7. However, the data from this cannot be analyzed properly because the

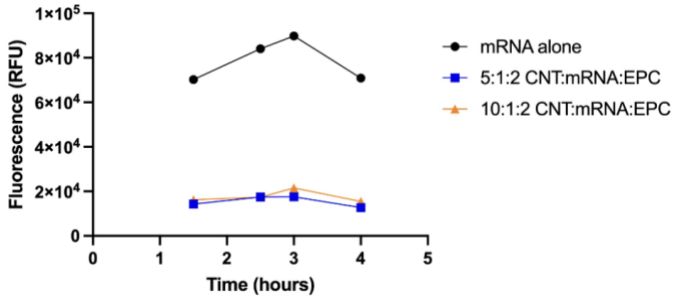

Fig. 7. IVT assay comparing *in-vitro* translation of mRNA alone and mRNA attached to CNT-PEI with EPC in Hela cell lysate at 5:1 and 10:1 mass ratios of CNT:mRNA.

calibration curve used for the first IVT assay could not be used for this assay. Therefore, we could not convert the fluorescence to concentration values. However, based on the fluorescence, the 5:1:2 and 10:1:2 CNT:mRNA:EPC ratio resulted in similar *in-vitro* translation. No statistical testing was performed due to the limitations of the analysis.

In-vitro Transfection Studies

Transfection studies were conducted to determine the cellular uptake of different formulations within Calu-3 cells. The first transfection study was performed to determine the effect of EPC on cellular transfection at a constant concentration of 0.4 ug mRNA. Figure 8shows the results of

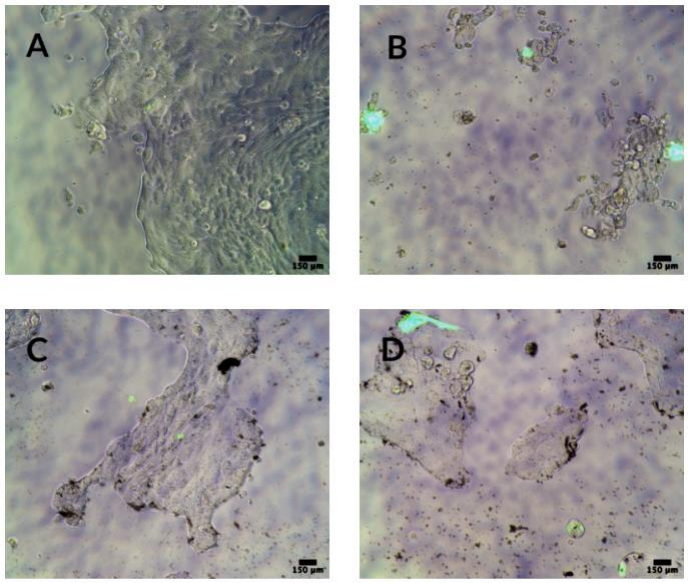

Fig. 8. Calu-3 Cells 48 Hours Post Transfection in a 96 Well Plate. **(A)** 1:1 CNT:mRNA without EPC, **(B)** 1:1 CNT:mRNA with EPC **(C)** 5:1 CNT:mRNA without EPC, **(D)** 5:1 CNT:mRNA with EPC

the first transfection study. EPC improved cellular uptake in both the 1:1 and 5:1 CNT:mRNA formulations. The second transfection study tested different mRNA doses in the ratios with EPC against a cells only and mRNA negative control. The mRNA concentrations tested were 0.5 ug and 1 ug for the 0:1:2, 1:1:2, and 5:1:2 ratios. Figure 9 shows the results of the second transfection study. Both 1:1:2 and 5:1:2 CNT:mRNA:EPC formulations in both mRNA concentrations showed cellular transfection as qualitatively compared to the cells only control. There was no fluorescence in the Lipofectamine positive controls.

Discussion

Interpretation of results

The loading efficiency indicated that the EPC improved loading of mRNA onto the CNTs. The 5:1 CNT:mRNA ratios resulted in better loading of the mRNA in both the EPC and no EPC cases. The first bonding affinity gel indicated that as the ratio of CNT:mRNA increased, bonding increased with the exception of the 1:1 ratio. Therefore, the 1:1, 5:1, and 10:1 ratio showed the best bonding. The second gel confirmed the results from the loading efficiency. The EPC did improve bonding between CNT and mRNA with the 5:1 and 10:1 showing the best results. The 1:1 and 5:1 ratios were used in future studies with cells.

The first IVT assay showed that translation was not hindered by the CNTs significantly when compared to mRNA alone. This was promising and led to the third IVT assay where the *in-vitro* translation of mRNA in different ratios was tested. This IVT assay showed similar *in-vitro* translation between the 5:1:2 and 10:1:2 CNT:mRNA:EPC samples. The cytotoxicity test suggests that CNTs are not toxic because all the samples without EPC were non-toxic among all CNT concentrations tested. It did show that the EPC may be more toxic at higher concentrations. Future studies could test different concentrations of EPC used in the ratios with the higher concentrations of CNTs. For example, a sample of 5:1:1.5 CNT:mRNA:EPC could be tested at a CNT concentration of 500 ug/mL. This could indicate the limit for increasing the amount of CNT with the least amount of EPC without sacrificing the bonding and loading of mRNA.

The first transfection study indicates that EPC is important for cellular uptake as the samples with EPC had more uptake than the samples without. The second transfection, as seen in Figure X, shows the ability for all CNT:EGFP mRNA:EPC formulations tested to be transfected into Calu-3 cells and translated into EGFP.

Selection of optimal formulations

The best ratios for bonding affinity were the 5:1:2 and 10:1:2 CNT:mRNA:EPC ratios as indicated by the gels. The best ratio for loading efficiency was 5:1:2 CNT:mRNA:EPC. The CNT-PEI concentrations that were non-toxic were all the samples without EPC and the 10 ug/mL and 1 ug/mL formulations. The IVT assays showed that CNTs do not hinder the *in-vitro* translation of mRNA as compared to mRNA alone. The transfection studies showed that the 1:1:2 and 5:1:2 ratios had successful transfection into the cells. From all these studies, the best formulation is determined to be the 5:1:2 CNT:mRNA:EPC ratio with a CNT concentration between 1 ug/mL and 100 ug/mL.

Sources of error and limitations

Lower initial masses of CNTs were used in the cutting and filtration process which resulted in a lower yield. For the remaining experiments, a pre-cut stock of CNTs was used. Three IVT assays were performed in

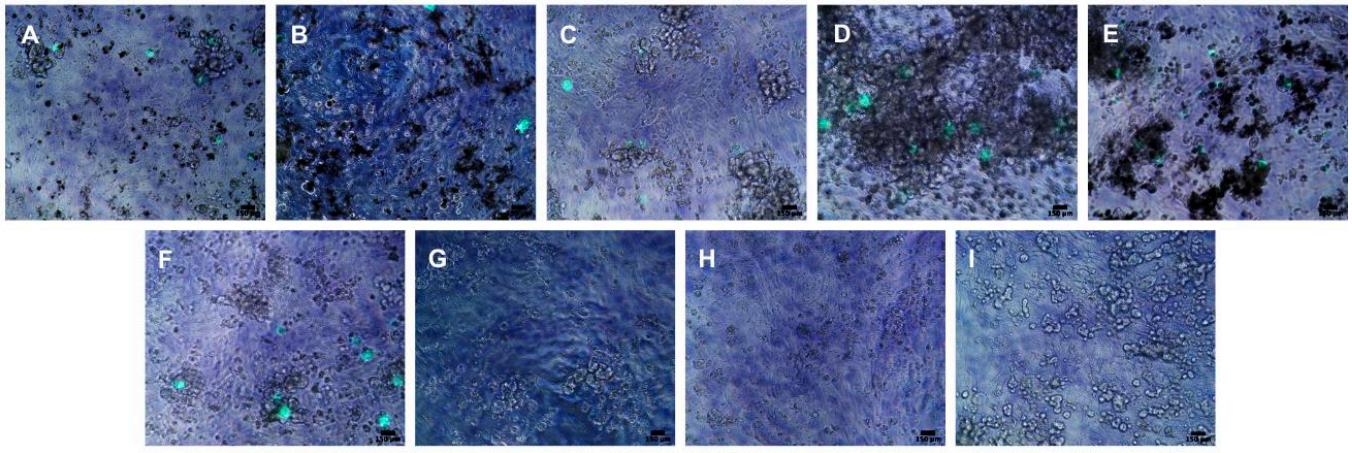

Fig. 9. Calu-3 Cells 48 Hours Post Transfection in a 96 Well Plate. **(A)** 0.5 ug of mRNA on CNT with a ratio of 1:1 CNT:mRNA with EPC, **(B)** 0.5 ug of mRNA on CNT with a ratio of 5:1 CNT:mRNA with EPC **(C)** 0.5 ug of mRNA:EPC with no CNTs, **(D)** 1 ug of mRNA on CNT with a ratio of 1:1 CNT:mRNA with EPC, **(E)** 1 ug of mRNA on CNT with a ratio of 5:1 CNT:mRNA with EPC, **(F)** 1 ug of mRNA:EPC with no CNTs, **(G)** 1 ug of mRNA:EPC with DNALipoMax, **(H)** 1 ug of mRNA:EPC with RNALipoMax, **(I)** Control well with cells only

total. However, the second IVT assay resulted in un-usable data because there were not enough reagents. In particular, there was not enough Hela lysate and Reaction Mix. This resulted in bubbly and evaporated wells. The third IVT assay resulted in usable data, but there was no calibration curve for higher concentrations of mRNA. Therefore, the fluorescence values could not be converted to concentration. Additionally, the loading efficiency is not 100% in the CNT:mRNA formulations, so there could be free-floating mRNA in the sample that is translating in the lysate rather than the CNT:mRNA complexes in the IVT studies. A limitation in the transfection studies was the lipofectamine control. Lipofectamine was used as a positive control. However, these samples did not result in any transfection as predicted. A theory for this is the potential cytotoxicity of lipofectamine. It can become toxic to cells after a few hours, but the study required measurement after 24-48 hours.

Future Work

Future work should focus on continuing the IVT, transfection studies, and cytotoxicity testing. The IVT assay comparing the different ratios should be performed again and tested against a calibration curve created with a higher concentration of mRNA. Suggestions to improve the IVT assays include washing the CNT formulations to wash out the free-floating mRNA in the sample. Transfection should be measured using flow cytometry so that different ratios can be quantitatively analyzed and compared. This will help determine the best ratio for transfection. For future transfection studies, Lipofectamine samples should be replaced with fresh media after a couple of hours to prevent cell death. Future transfection studies should also be conducted in Transwell plates to determine if uptake and translation can still occur if the formulation needs to pass a barrier as it would *in vivo*. In the transfection studies done in this experiment, mRNA easily transfected cells because it did not have to travel far.

Future cytotoxicity studies should test different concentrations of EPC used in the ratios with the higher concentrations of CNTs because EPC showed potential toxicity. For example, a sample of 5:1:1.5 CNT:mRNA:EPC could be tested at a CNT concentration of 500 ug/mL. This could indicate the maximum amount of CNT with the least amount of EPC that does not sacrifice the bonding and loading of mRNA. Additionally, LDH assays should be run again with mRNA added to the formulations. While it was determined previously that EGFP mRNA is not toxic to the cells, the addition of mRNA to the formulation may result in unforeseen toxic effects. A calcein AM/ethidium homodimer-1 (CaAM/EthH) staining and/or carrier digestion and trypan blue (TB) assay should be performed to verify the results of the LDH assay. Each of these assays assess cytotoxicity in different manners. One of the main concerns for this vaccine delivery method is the lack of knowledge about the toxicity of the CNTs. Performing different types of cytotoxicity tests can create a complete picture about this unknown factor of CNTs. After these initial tests, the 5:1:2 CNT:mRNA:EPC formulation should be tested in animal models. Animal testing should include toxicity tests and efficacy of the formulation to prevent HIV.

Materials and Methods

Cutting

Purified multi-walled carbon nanotubes (MWCNTs) of 60-100 nm diameter were obtained from SES Research and 3 mg were measured and placed into a polypropylene tube. A 3:1 ratio of sulfuric acid (H_2SO_4) and nitric acid (HNO_3) was added to the tube containing CNTs to cut them. The tube was then placed in a beaker of water and sonicated at 40°C overnight until the solution turned brownish yellow. Ice was added to keep the temperature below 50°C. A container was placed on a magnetic plate and filled with 1L of DI water and a stir bar. The CNT solution was poured into the container and 6M NaOH was added until the pH stabilized between 7.0 and 8.0. HCl was added to adjust the pH as necessary. Once a stable pH of ~7.5 was reached, the solution was covered with foil and left stirring overnight.[9,13]

Separation

CNTs were separated to obtain short CNTs only in the target size range. The solution was filtered through a 0.05 um filter using an Erlenmeyer vacuum filter flask attached to a vacuum pump. CNTs and salts were left dry on the filter and gathered into a beaker using tweezers and DI water. This beaker was sonicated for 30 minutes until all CNTs were dispersed in solution. This solution was then filtered using the same apparatus but with a 0.4 um filter to remove any large CNTs or salts.[9,13]

Purification

The solution from the Erlenmeyer flask after the second filtration is then added to a centrifugal filtration tube and centrifuged for 5 minutes at 6000 RPM using the Thermo Scientific IEC CL31 Multispeed Centrifuge. The liquid waste from the bottom of the filter tube was removed. This step was repeated until all CNTs were filtered. DI water and a pipette were then used to remove all of the CNTs off of the filter. The CNTs were resuspended in 1 ml of DI water to create a 3 mg/ml solution.[9,13]

CNT-PEI Conjugation

The 3 mg/ml CNT solution from the previous step was combined with 1.1 ml of 10X MES buffer. To activate the CNTs, 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide (EDC) and N-hydroxysulfosuccinimide (Sulfo-NHS) were weighed out at 1 mg and 2.75 mg, respectively, and transferred to the CNT solution. The tube was vortexed for 1 minute and then incubated for 30 minutes at room temperature to allow the reaction to occur. The CNTs were then purified by centrifugal filtration 3 times using DI water to remove any excess EDC and Sulfo-NHS. To conjugate CNT with PEI25K, 1.1 ml of 10X PBS solution was added to the activated CNTs. Then, 30 mg of linear polyethylenimine with a molecular weight of 25,000 (PEI25K) was added to the solution and mixed by vortexing to make a 3 mg/mL solution of CNT-PEI25K. The sample was left at room temperature overnight to allow the reaction to occur. The CNT-PEI solution was then purified once more by completing 3 rounds of centrifugal filtration using DI water to remove any excess PEI25K. The final solution was stored in a microcentrifuge tube wrapped in aluminum foil and placed in a 4C refrigerator.[9,13]

CNT Characterization

The size and charge of short CNTs were measured using Dynamic Light Scattering and Zeta Potential Analysis (Zetasizer Nano ZS90, Malvern Instruments, Inc) respectively before and after PEI conjugation.

Loading efficiency

The loading efficiency of EGFP-mRNA was measured using a spectrophotometer. Several vaccine formulations were measured, including 5:1:0, 5:1:2, 1:1:0, and 1:1:2 mass ratios of CNT:mRNA:EPC. Two mRNA only controls were also included at mass ratios of 0:1:0 and 0:1:2 CNT:mRNA:EPC. The formulations were placed in microcentrifuge tubes and spun down for 30 minutes at 14,000 rpm until all CNT-mRNA complexes formed sufficient pellets at the bottom of the tube. The spectrophotometer was blanked with DI water and the supernatant from each formulation was measured to determine the unbound mRNA concentration. The loading efficiency (LE) was calculated using the following equation:

$$LE = 100[\frac{mRNA\ \ only\ conc. - supernatant\ mRNA\ conc.}{mRNA\ only\ conc.}] \quad [1]$$

Formulations containing EPC used mRNA-EPC only as their control and formulations containing no EPC used mRNA only as their control.[9,13]

Bonding affinity

The bonding affinity of CNT to EGFP mRNA was measured using gel electrophoresis. A 1% agarose gel was prepared using Tris-acetate-EDTA (TAE) buffer. The solution was microwaved and stirred until agarose was fully dissolved. SYBR Safe DNA gel stain was added to the solution and poured into an Owl Model B1A gel rig with 1.5 mm 10-well comb and cooled until solid. Several gels were run to test to determine the best bonding affinity between CNT and mRNA. For each gel run, CNTs were sonicated for 30 minutes before sample preparation and the ladder samples were prepared with the Thermo SM1823 ladder. The concentration of mRNA in all samples for each gel run was 200 ng. The samples tested in the first gel run included CNT:mRNA mass ratios of 0:1, 1:5, 1:1, 1:2, 2:1, and 5:1. The

samples tested in the second gel included CNT:mRNA mass ratios of 0:1, 1:1, 2:1, 3:1, 5:1, 7.5:1, and 10:1. The samples tested in the third gel included CNT:mRNA:EPC mass ratios of 0:1, 1:1, 1:1:2, 5:1, 5:1:2, 10:1, 10:1:2, and 0:1:2. Samples were incubated for 15 minutes to allow mRNA to bond to CNTs. The gels were run for 2 hours at 50V and imaged using fluorescence imaging.[9,13]

Cell Culture

A vial of passage 1 (P1) Calu-3 cells were thawed, combined with complete culture media (EMEM with 10% FBS) and spun down at 125g for 5 minutes. The cell pellet was resuspended in 1 ml and combined with 14 more ml of complete growth media and then placed in a T75 flask. The cells grew for 8 days at 37°C until they became confluent. The cells were then incubated with 5 ml of 0.25% w/v Trypsin and 0.53 mM EDTA at 37°C to detach cells from the surface of the flask. This incubation occurred for approximately 30 minutes until observation under a microscope indicated that most of the cells had detached. Following detachment, 15 ml of complete growth media was added to the flask to deactivate the Trypsin-EDTA solution. The cells were then spun down at 125g for 5 minutes, resuspended in 1 ml of complete growth media, and counted using an Invitrogen Automated Cell Counter. Calculations were performed to determine cell seeding densities. The cells were then seeded in a 96 well plate for future experiments.[9,13]

Cytotoxicity

A lactate dehydrogenase (LDH) assay was prepared to determine cytotoxicity levels of CNTs cultured with Calu-3 cells. Calu-3 cells were incubated in triplicate with CNTs at concentrations of 500, 100, 10, and 1 ug/ul with and without EPC. Cells containing complete media served as a negative control, while samples containing Triton-X served as a positive control. Several other controls that did not contain cells were also incorporated to determine background fluorescence, including basal media, complete media, along with each of the CNT concentrations. After an overnight incubation, the supernatants from each well were removed and placed into their own respective microcentrifuge tubes. The remaining cells in the plate were lysed with Triton-X and placed them into separate microcentrifuge tubes. These samples were frozen down at -4°C overnight and thawed at room temperature the next day. To run the LDH assay, 50 uL of each respective supernatant and lysed sample were added to individual wells in another 96 well plate. Then, 50 ul of the LDH Reaction Mixture was added to each well. After 20 minutes of incubation, 50 ul of Stop Solution was added, and the absorbance was measured using a BioTek Cytation 5 microplate spectrophotometer at 490 and 680 nm. Values from the 680 nm reading were subtracted from the 490 nm reading as done in previous protocols. Averages and standard deviations were then calculated from the triplicates for each group. Cytotoxicity was measured relative to a negative toxicity control at 0%. The following equation was used:

$$\%\,Toxicity = 100[\frac{\text{Sample LDH} - \text{Background LDH}}{\text{Negative toxicity lysed LDH}}] \quad [2]$$

Background LDH is the LDH intensity measured from cells only in complete media. Sample LDH is the intensity of LDH measured in each respective sample group. Negative toxicity lysed LDH is the expected amount of LDH in a healthy cell population.[9,13]

In-vitro Translation (IVT)

A 96-well plate of Calu-3 cells was prepared for the IVT assay. Reagents from the Thermo Scientific 1 Step Human Coupled DNA IVT Kit were thawed. The reaction was prepared at room temperature by combining HeLa lysate, accessory proteins, Reaction Mix, and nuclease free water in microcentrifuge tubes using proportions specified in the protocol. The first run of IVT contained EGFP-mRNA at a concentration of 0.09 ug/uL at a 1:1 mass ratio of CNT:mRNA. The second and third runs of IVT contained EGFP-mRNA at a concentration of 0.5 ug/uL and included vaccine formulations of 10:1:2 and 5:1:2 mass ratios of CNT:mRNA:EPC along with an mRNA only control and a HeLa lysate control that included accessory proteins and Reaction Mix. Each formulation and control was then added into a 96-well plate. The reaction was incubated for 4 hours with measurements taken at 1.5, 2.5, 3, and 4 hours on a plate reader at 475 and 520 nm. A CNTVac EGFP standard curve was used to convert fluorescence intensities into concentrations of EGFP in ug/uL. The following equation was used for conversion:

$$y = 17179 - 9898\text{x} + 1340x^2 \quad [3]$$

where y is fluorescent intensity and x is concentration.[9,13]

Transfection/Cellular Uptake

Transfection studies were performed to determine cellular uptake of different formulations within Calu-3 cells, a mucosal cell line. For the first run of transfection, after cells reached approximately 80% confluency in a T75 flask, they were seeded in a 96-well plate at a density of $1x10^5$ cells per well. The following day, each well was replaced with fresh EMEM media containing 10% FBS. Several vaccine formulations were prepared including CNT:mRNA:EPC ratios of 1:1:2, 1:1:0, 5:1:2, and 5:1:0 at an mRNA concentration of 0.4 ug/ul, along with an mRNA only control and a cells only control. Lipofectamine MessengerMAX Transfection Reagent from Invitrogen was used as a positive control. Each formulation and control was diluted in OptiMEM media. The cells were transfected in triplicate with each vaccine formulation and control and incubated for 30 hours at 37°C. Not much fluorescence was visualized, so the plate was incubated for another 72 hours and then visualized. The second run of transfection followed a similar protocol, but included ratios of 1:1:2, 5:1:2, and 0:1:2 CNT:mRNA:EPC at 0.5 ug and 1 ug concentrations of mRNA. The cells were seeded at a density of $2x10^5$ cells per well and samples were transfected in triplicate. Positive controls consisted of mRNA with Lipofectamine Transfection Reagent at 0.5 and 1 ug concentrations, along with mRNA with Lipofectamine MessengerMAX at a concentration of 1 ug. A negative control containing cells only was also incorporated. The second plate was incubated for 48 hours. Both runs of transfection were imaged using brightfield and fluorescence microscopy and analyzed qualitatively through observance of fluorescence intensity.

End Matter

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